

From the Department of Women's and Children's Health  
Karolinska Institutet, Stockholm, Sweden

# **METABOLIC EFFECTS AND LONG-TERM SAFETY OF CHILDHOOD GROWTH HORMONE TREATMENT**

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Institutet**

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# METABOLIC EFFECTS AND LONG-TERM SAFETY OF CHILDHOOD GROWTH HORMONE TREATMENT THESIS FOR DOCTORAL DEGREE (Ph.D.)

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“Children are either too short or too tall, too fat or too lean. Their adolescence is too early or too late: they have too little or too much hair. They are intellectually backward or stupid, even defective or epileptic. The sella turcica is too small or too large and its bedposts are of the wrong shape or may even coalesce. The pineal gland casts a shadow and must be causing trouble. The basal metabolism, laboriously calculated, is found to be a little low or a little high. All this needs attention and can be corrected by some whole-gland extract, usually with a pinch of thyroid thrown in.”

*Harvey Cushing in Disorders of the pituitary gland: Retrospective and prophetic (J Am Med Assoc, 1921;76:1721-1726)*

*To Liselotte, Clara, Agnes and Erik*



## ABSTRACT

The overall aim of this thesis was to investigate the metabolic effects and long-term safety of childhood growth hormone (GH) treatment. In order to achieve this aim, the different projects consist of a two-part clinical trial on metabolic features linked to GH physiology and GH treatment as well as two large population-based cohort studies with focus on the long-term cardiovascular and cancer risks in previously GH-treated patients.

In **study I**, the metabolic profile of 35 prepubertal children of short stature, between 7 and 10 years of age, with stimulated peak GH levels in the lower normal range (7-14  $\mu\text{g/L}$ ) was compared to 12 age- and sex-matched control children of normal height and weight. The groups were compared using blood samples of fasting glucose and insulin, HbA1c, insulin-like growth factor I (IGF-I), insulin sensitivity using both homeostasis model assessment of insulin resistance (HOMA-IR) and frequently sampled intravenous glucose tolerance test (FSIVGTT), dual-energy x-ray absorptiometry (DEXA), microdialysis and stable isotope examination of glucose production and lipolysis. Few differences between the groups were found but the subgroup of children with the lowest GH peak levels demonstrated lower fasting insulin levels and signs of increased insulin sensitivity.

In **study II**, the 35 short children from study I were subsequently randomized to three different doses of recombinant human GH (rhGH) treatment; low dose (11  $\mu\text{g/kg/d}$ ), standard dose (33  $\mu\text{g/kg/d}$ ) or high dose (100  $\mu\text{g/kg/d}$ ), and followed for two years. The doses were blinded to both patients and the study investigators. The metabolic effects of the different treatment doses were analyzed by the same methods as in study I and a clear dose-dependent metabolic effect could be demonstrated, in particular for the high dose group regarding fasting insulin and different measures of insulin sensitivity.

In **study III**, the long-term cardiovascular morbidity in childhood rhGH-treated Swedish patients between 1985 and 2010, due to isolated GH deficiency (GHD), small for gestational age (SGA) or idiopathic short stature (ISS), was investigated. Data on cardiovascular outcomes and important covariates were gathered for a total of 3,408 patients and 50,036 randomly selected controls matched on sex, age and county. Time to first cardiovascular event was analyzed by Cox proportional-hazard regression models and the study showed increased adjusted hazard ratios for the patients compared to the controls.

In **study IV**, the long-term cancer incidence and mortality in a large meta-cohort of approximately 24,000 previously childhood rhGH-treated patients from eight European countries were investigated. The results did not support an overall carcinogenic effect of rhGH treatment but the significant trend of increased cancer mortality risk in relation to rhGH dose in patients with previous cancer and the indication of possible effects on bone cancer, bladder cancer and Hodgkin's lymphoma requires further vigilance.

## LIST OF SCIENTIFIC PAPERS

- I. **Tidblad A**, Gustafsson J, Marcus C, Ritzén M, Ekström K.  
Metabolic differences between short children with GH peak levels in the lower normal range and healthy children of normal height.  
*Growth Hormone & IGF research: official journal of the Growth Hormone Research Society and the International IGF Research Society*. 2017; 34:22-27
- II. **Tidblad A**, Gustafsson J, Marcus C, Ritzén M, Ekström K.  
Metabolic effects of Growth Hormone treatment in short prepubertal children: a double-blinded randomized clinical trial.  
*Manuscript*
- III. **Tidblad A**, Bottai M, Kieler H, Albertsson-Wikland K, Säwendahl L.  
Childhood Growth Hormone Treatment and Long-Term Cardiovascular Morbidity.  
*Manuscript*
- IV. Swerdlow AJ, Cooke R, Beckers D, Borgstrom B, Butler G, Carel JC, Cianfarani S, Clayton P, Coste J, Deodati A, Ecosse E, Gausche R, Giacomozzi C, Hokken-Koelega ACS, Khan AJ, Kiess W, Kuehni CE, Mullis PE, Pfaffle R, Säwendahl L, Sommer G, Thomas M, **Tidblad A**, Tollerfield S, Van Eycken L, Zandwijken GRJ.  
Cancer Risks in Patients Treated With Growth Hormone in Childhood: The SAGhE European Cohort Study.  
*The Journal of Clinical Endocrinology and Metabolism*. 2017; 102:1661-1672

### Related publications not included in the thesis:

Swerdlow AJ, Cooke R, Beckers D, Butler G, Carel JC, Cianfarani S, Clayton P, Coste J, Deodati A, Ecosse E, Hokken-Koelega ACS, Khan AJ, Kiess W, Kuehni CE, Fluck CE, Pfaffle R, Säwendahl L, Sommer G, Thomas M, **Tidblad A**, Tollerfield S, Zandwijken GRJ.  
Risk of Meningioma in European Patients Treated With Growth Hormone in Childhood: Results From the SAGhE Cohort.  
*The Journal of Clinical Endocrinology and Metabolism*. 2019; 104:658-664



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## LIST OF ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
AER	Absolute excess risk
AIR	Acute insulin response
AITT	Arginine and insulin tolerance test
ALS	Acid labile subunit
BMI	Body mass index
CI	Confidence interval
CJD	Creutzfeldt-Jakob Disease
DEXA	Dual-energy x-ray absorptiometry
EMA	European Medicines Agency
FDA	Food and Drug Administration
FFA	Free fatty acids
FSIVGTT	Frequently sampled intravenous glucose tolerance test
FSS	Familial short stature
GH	Growth hormone
GHBP	Growth hormone binding protein
GHD	Growth hormone deficiency
GH <sub>max</sub>	Growth hormone peak level
GHR	Growth hormone receptor
GHRH	Growth hormone releasing hormone
GHS-R	Growth hormone secretagogue receptor
GLUT	Glucose transporter
hGH	Human growth hormone
hGH-V	Variant of human growth hormone
HL	Hepatic lipase
HOMA-IR	Homeostasis model assessment of insulin resistance
HR	Hazard ratio
HSL	Hormone-sensitive lipase
ICD	International classification of diseases
IGF-I	Insulin-like growth factor I

IGF1R	Insulin-like growth factor I type 1 receptor
IGFBP	Insulin-like growth factor binding protein
IRR	Incidence risk ratio
IRS	Insulin receptor substrate
ISS	Idiopathic short stature
JAK	Janus kinase
LPL	Lipoprotein lipase
MPHD	Multiple pituitary hormone deficiency
NSILA	Non-suppressable insulin-like activity
PI3K	Phosphoinositide-3-kinase
Pyrs	Person-years
rhGH	Recombinant human growth hormone
SAGhE	Safety and Appropriateness of Growth hormone treatments in Europe
SDS	Standard deviation score
SGA	Small for gestational age
Si	Insulin sensitivity index
SIR	Standardized incidence ratio
SMR	Standardized mortality ratio
STATs	Signal transducers and activator of transcription proteins
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TACE	Tumor necrosis factor- $\alpha$ -converting enzyme

# 1 INTRODUCTION

Growth hormone (GH) is produced by the somatotrophic cells of the anterior pituitary gland and has numerous important physiological effects concerning growth and metabolism in all vertebrate species. In humans, different pathological states of deficiency or excess of GH are found in both adults and children, which can consequently lead to disturbances of growth as well as other metabolic functions in the body.

A common patient group within the field of pediatric endocrinology are children with short stature, in which the question of possible GH deficiency and treatment with GH is often raised. Several hundreds of thousands of children have been treated with GH worldwide, since its introduction more than fifty years ago, and the treatment indications have gradually expanded from the most severe cases of GH deficiency, when treatment was scarce, to at present include several conditions in which the associated short stature is not primarily thought to be due to deficient endogenous GH secretion.

My own interest in this field started from a general interest in physiology and a more specific interest in growth and metabolism based on my clinical work with this patient group. An understanding of the widespread effects of GH on metabolism and its efficacy and potency of treating short stature, regardless of the underlying cause, also triggered my interest in the safety issues related to this treatment. The different projects included in this thesis are therefore focused on these topics with the attempt to increase our current understanding of the metabolic effects linked to GH as well as our knowledge of the long-term safety of childhood GH treatment.

Even if many patients have been treated with GH to this date, the current evidence of its long-term safety is still uncertain due to the lack of long-term follow-up studies. The treatment effect on longitudinal growth has been extensively described over the years and large post-marketing surveillance studies have suggested its short-term safety. However, the long-term safety of childhood GH treatment is still, to a large extent, uncertain and a central aim of this thesis is to bring more clarity to this issue.



## 2 BACKGROUND

### 2.1 HISTORICAL BACKGROUND

The conception of a pituitary-derived substance and its relation to growth dates back to the late 19<sup>th</sup> century. In 1886, the French neurologist Pierre Marie described a condition with abnormal growth of the hands, feet and the face which he proposed to call “acromegaly”, from the Greek words for extremities (“akro-”) and enlargement (“megalos”).<sup>1</sup> Earlier description of this condition can be traced even further back to the 16<sup>th</sup> and 17<sup>th</sup> century with several names given to describe its features, such as “géant scrofuleux”, “prosopo-ectasia” or “macrosomia”.<sup>2</sup> Marie continued his work to meticulously describe the phenotype of acromegaly but the etiology was still largely unknown, even if he pointed out that all cases had a “hypertrophy of their pituitary body with enormous dilatation of the Sella turcica”.<sup>3</sup> This observation was also supported by others, such as Dr. Oskar Minkowski in 1887.<sup>4</sup>

Gradually during the coming years, several descriptions of abnormalities in the sella region was correlated to this condition and in 1892 the physician Roberto Massalongo could further describe the association with increased pituitary function in an acromegaly patient shown to have a pituitary tumor containing specific granulated cells.<sup>5</sup>

However, it was not until the beginning of the 20<sup>th</sup> century that an agreement regarding the pathogenesis of acromegaly developed, in which it was attributed to hyperactivity of the pituitary gland and that gigantism had the same pathogenic mechanism but developed earlier in life before the epiphyseal fusion.<sup>6</sup>



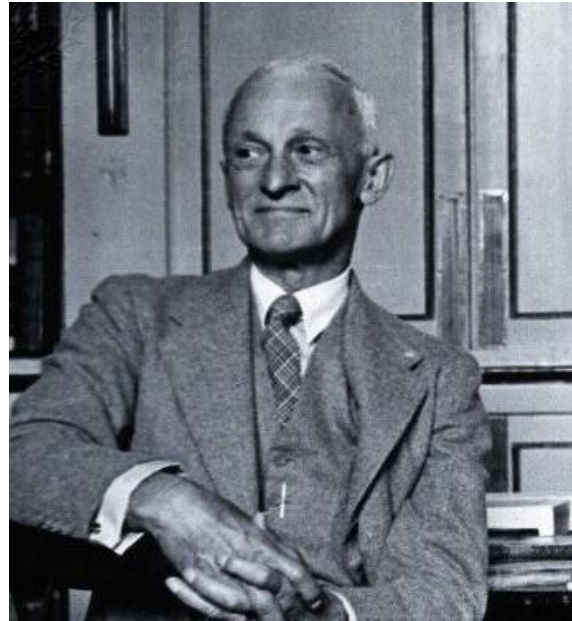
**Figure 1.** The two French brothers with gigantism (measuring 230 cm) posing with a person with pituitary dwarfism (measuring 69 cm) in a historical postcard

*Source: Reproduced from Enderle A. Dwarfism and gigantism in historical picture postcards. J R Soc Med. 1998;91:273-278.*

Around the same time as the understanding of the pathogenesis of acromegaly and gigantism developed, the connection of extreme short stature (dwarfism) and pituitary dysfunction was suggested independently by Hutchinson and Benda in 1900.<sup>7</sup> The idea that the same cells that created acromegaly or gigantism if overactive, also led to dwarfism if underactive, was further suggested by the Austrian physiologist Bernard Aschner<sup>8</sup> and the American

neurosurgeon Harvey Cushing coined the terms hyper- and hypopituitarism in 1909<sup>9</sup> and referred to “the hormone of growth” in his celebrated monography *The Pituitary Body and its Disorders* from 1912.<sup>10</sup>

Aschner published extensive studies on 20 hypophysectomized dogs with a clear description of growth arrest in these animals and became convinced that the pathogenesis of acromegaly must be caused by a hyper-function of the pituitary gland rather than hypo-function that had been suggested earlier by several other authors. In addition, Cushing’s systematic exploration of the pituitary gland and its function, in both clinical cases and animal experiments, contributed greatly to expand the scientific knowledge of this gland, its hormones yet to be discovered, and its interaction with the hypothalamus.<sup>11</sup>



**Figure 2.** Harvey Cushing in 1938

Source: Wellcome Trust Library

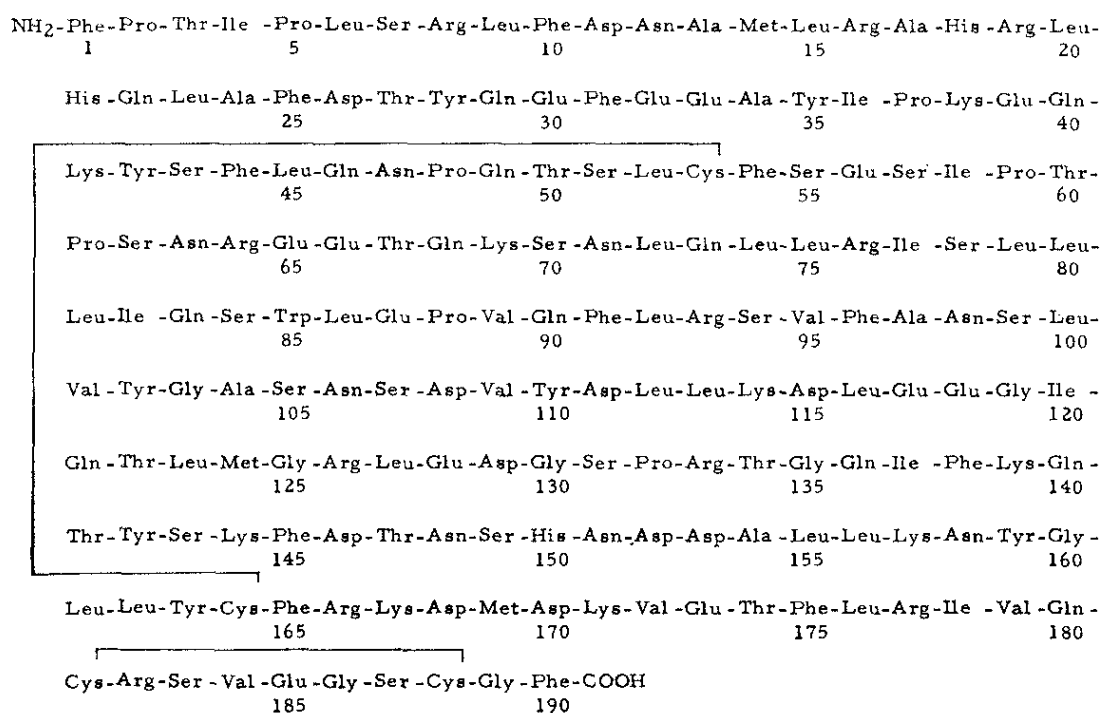
In the coming decade, advances in the understanding of the connection between the pituitary gland and growth disorders continued through experimental hypophysectomies on different animal models. In tadpoles, Dr. Philip Smith reported an almost complete cessation of growth and also the reduction in size of the thyroid and gonads in the hypophysectomized animals.<sup>12</sup> In a later issue of *Science* the same year (1916), Bennet Allen described experiments with similar findings of growth retardation, change in pigmentation and striking retardation of limb development.<sup>13</sup> In the following years, Smith could further demonstrate that the phenotype could be reversed by grafts of the anterior pituitary lobe and later also by extracts from bovine anterior pituitary lobes.<sup>7</sup>

Perhaps this innovative work of Smith at the Anatomy department of the University of California in Berkeley inspired the head of the department, professor Herbert M. Evans, to take interest in the field. Together with his associate Joseph Long, Evans published a classical and groundbreaking study in 1921 in which they established the growth-promoting effects in rats by administration intraperitoneally of “finely ground, fresh anterior lobe of the hypophysis of beef”.<sup>14</sup> At the 333<sup>rd</sup> day of life the treated rats were more than twice as heavy as the control animals and the authors concluded that “it would not appear to be incorrect to characterize these changes as producing constantly a certain degree of true gigantism”.<sup>14</sup> They also demonstrated that no effect was seen if this was given orally or if they received posterior hypophyseal substance instead.



Some years later, in 1927, Smith could further support these findings in rats by demonstrating that hypophysectomy induced the “pituitary syndrome”; including growth inhibition (in the young) and cachexia (in the adult), atrophy of the genital system, loss of libido, atrophy of the thyroid, parathyroid and suprarenal cortex as well as general weakness and physical impairment.<sup>15</sup> He could thereafter completely reverse this picture by transplanting gland material from adult rats intramuscularly with an “immediate and striking” response.<sup>15</sup>

It would take an additional 20 years of extensive work before Evans together with his associate the biochemist Choh Hao Li finally in 1944 could isolate the substance from the anterior pituitary gland which promoted growth in hypophysectomized animals.<sup>16</sup> They could also demonstrate in their female rats that the “the product did not show lactogenic, thyrotropic, adrenocorticotropic, follicle-stimulating or interstitial-cell stimulating activities, indicating that the preparation was substantially free of other biologically active pituitary contaminants”.<sup>16</sup> Li continued to dedicate his life to the purification and determination of the molecular structure of several of the hormones from the pituitary gland<sup>17</sup> and could in 1971 together with Jonathan Dixon publish the primary molecular structure of growth hormone.<sup>18</sup>



**Figure 3.** The amino acid sequence of the human growth hormone molecule published by Li and Dixon in 1971

Source: Reprinted from *Archives of biochemistry and biophysics*, 1971;146. Li CH, Dixon JS. Human pituitary growth hormone XXXII. The primary structure of the hormone: revision, pp. 233-6, Copyright (1971), with permission from Elsevier.

## 2.2 THE BIOCHEMICAL STRUCTURE, REGULATION AND SIGNALING OF GROWTH HORMONE

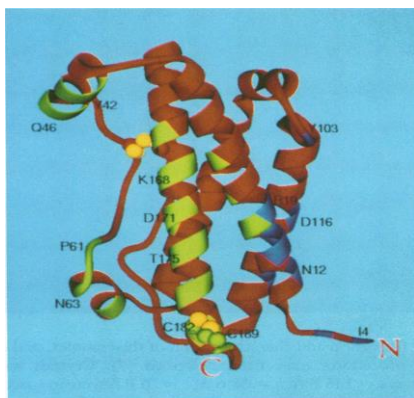
### 2.2.1 The human growth hormone gene

The human GH gene (*GH1*) is part of a cluster of five similar genes connected to growth on the long arm of chromosome 17 (17q23.3) and expressed in the somatotrophic cells of the anterior pituitary gland.<sup>19</sup> The gene *GH2* is situated at the same locus and encodes for a variant of hGH (hGH-V) exclusively expressed in the syncytiotrophoblasts of the placenta during fetal development.<sup>20</sup> The remaining homologous genes in the region, *CSHP* (chorionic somatomammotropin pseudogene), *CSH-1* and *-2* (chorionic somatomammotropin gene), are also exclusively expressed in placental tissue and act in concert with hGH-V to stimulate the development and growth of the fetus.<sup>19,21</sup>

The expression of *GH1* is regulated by a proximal promotor region with a high level of single nucleotide polymorphism and a locus control region located between 14.5 to 32 kb upstream from *GH1*.<sup>22</sup> Moreover, numerous transcriptional factors, coded by genes such as *POU1F1*, *PRO1* or *OTX2*, are involved in the development of the pituitary gland and different mutations in these genes have been shown to disturb its normal development and function.<sup>23</sup>

### 2.2.2 The biochemical structure of human growth hormone

The main form of human growth hormone (hGH) consists of 191 amino acids in a single chain polypeptide with a molecular weight of 22-kilodalton (kDa). The major structural features are its four  $\alpha$ -helices, hydrophobic core, and two disulphatic bonds between cysteine at position 53 and 165 and cysteine at position 182 and 189.<sup>24,25</sup> Other isoforms of hGH exist by alternative mRNA splicing of the 217-amino acid GH-precursor, such as a 20-kDa isoform lacking residues 32 to 46.<sup>26</sup> The principle 22-kDa isoform constitutes approximately 90% of the different isoforms in the circulation and it is still unclear if the other forms of hGH has any substantial functional difference.<sup>27,28</sup>



**Figure 4.** The three dimensional structure of human growth hormone published in Science by de Vos *et al* in 1992

Source: From de Vos A, Ultsch M, Kossiakoff A. Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. *Science* (New York, NY) 1992;255:306-12. Reprinted with permission from AAAS.

### 2.2.3 The regulation of growth hormone secretion

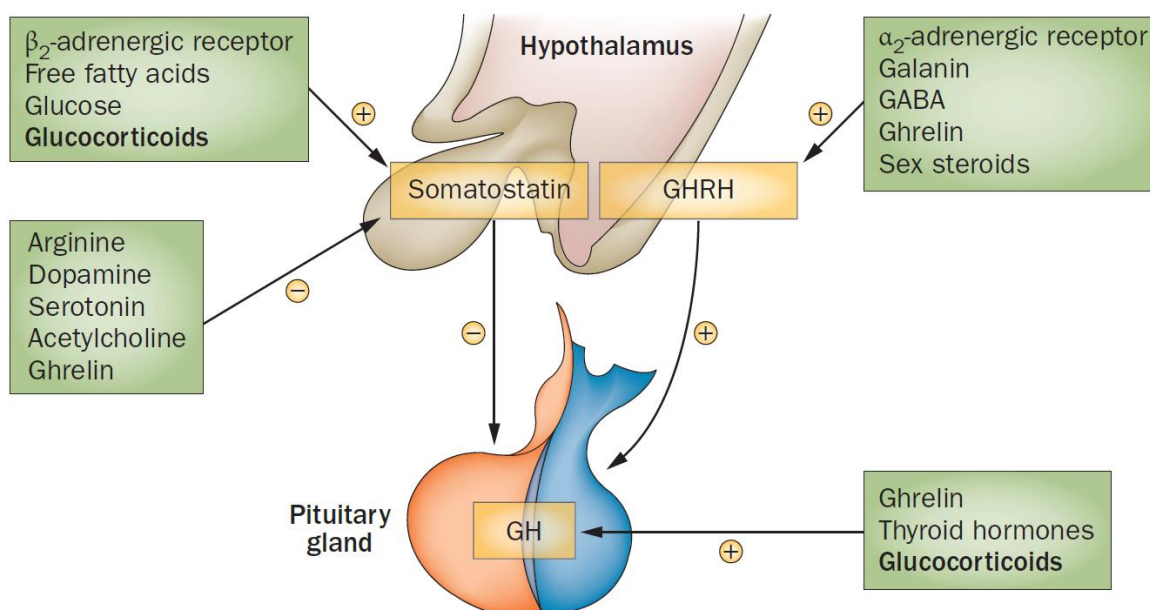
GH is secreted from the anterior pituitary gland in a pulsatile fashion approximately every three hours, predominantly during the night, with maximum peaks shortly after the first onset of slow-wave sleep (stages III and IV).<sup>29,30</sup> The secretion pattern is under hypothalamic control by the stimulatory peptide GH releasing hormone (GHRH) and the inhibitory counterpart somatostatin.<sup>31</sup> GHRH stimulates both synthesis and release of preformed GH but somatostatin only inhibits its release.<sup>31</sup> The troughs (low points) of GH release is to a large extent affected by the “somatostatin tonus” and its disinhibition is a permissive factor for the timing and amplitude of the recurrent GH pulses.<sup>32</sup>

In 1999, an additional important stimulatory peptide was discovered, ghrelin (the name given from the proto-Indo-European word “ghre” meaning growth), which stimulates GH release both directly and indirectly through GHRH and somatostatin.<sup>33</sup> The 28 amino acids long peptide was identified as the ligand to the already known GH secretagogue receptor (GHS-R) and was found to mainly be expressed in the stomach but also to some degree in the hypothalamus.<sup>34</sup> In high doses, ghrelin can additionally stimulate release of other pituitary hormones (ACTH, prolactin) and is involved in several other physiological processes, such as stimulating appetite, gastric acid secretion, gastric motility, and insulin secretion if coupled with high blood glucose levels.<sup>35</sup>

The secretion of GH is also regulated by a classical negative feedback loop by its prime mediator of its physiological effects, insulin-like growth factor I (IGF-I), which inhibits GH secretion both directly at the pituitary level<sup>36,37</sup> and indirectly by affecting GHRH and somatostatin signaling at the hypothalamic level<sup>38,39</sup>. GH have, in addition, a negative effect on its own secretion by inhibiting GHRH expression at the hypothalamic level.<sup>40,41</sup>

Many other factors also contribute to the complex regulation of GH release, ranging from a number of individual and environmental factors, such as stress<sup>42</sup>, exercise<sup>43</sup>, hypoglycemia<sup>44</sup>, fasting<sup>45</sup>, obesity<sup>46</sup>, age<sup>47</sup>, to other hormonal factors, such as the influence of sex steroids<sup>48</sup>, leptin<sup>49</sup>, glucocorticoids<sup>50</sup>, and more.

A schematic picture including some of the major factors regulating GH secretion is presented below.



**Figure 5.** Schematic overview of the regulation of growth hormone secretion by some of the described central and peripheral neuroendocrine signals

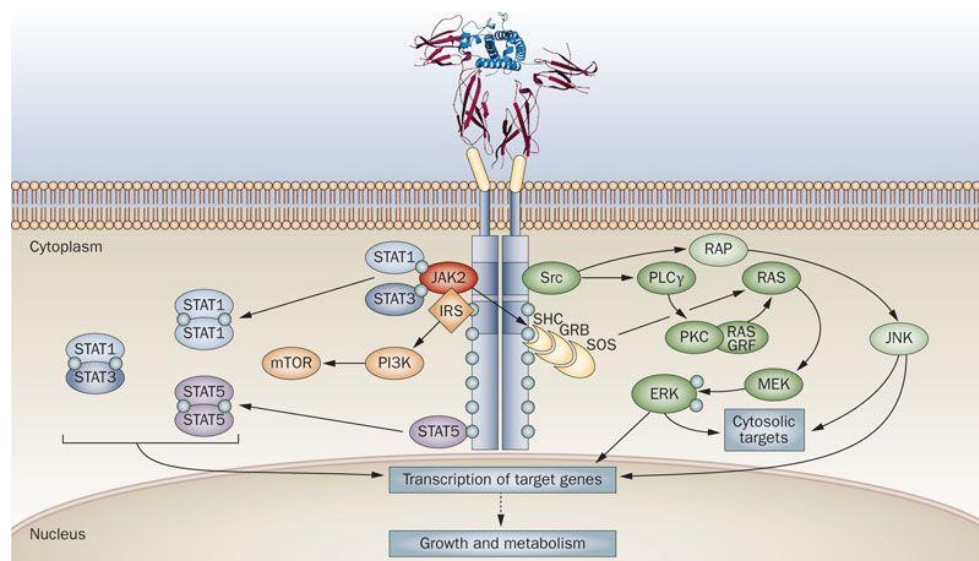
Source: Reprinted by permission from Springer Nature: *Nature Reviews Endocrinology, Glucocorticoids and the regulation of growth hormone secretion*, Mazziotti G, Giustina A, Copyright (2013).

## 2.2.4 Growth hormone signaling

The intracellular effects of GH are initiated through the binding to its cell surface receptor; the growth hormone receptor (GHR). The GHR is considered an archetypical cytokine receptor<sup>51</sup> being the first cytokine receptor to be purified and cloned in 1987 by Leung *et al.*<sup>52</sup> The receptor is a protein of 620 amino acids with an extracellular domain of 246 amino acids, a single 24 amino acid transmembrane helix and an intracellular domain consisting of 350 amino acids.<sup>52,53</sup> The gene coding for GHR is located on chromosome 5 (p13.1-p12), contains 10 exons of which nine are coding, and is expressed in many tissues but most abundantly in the liver.<sup>24,54,55</sup>

A circulating binding protein for GH (GHBP) was identified in 1986, just a year before the discovery of the GHR, and was later found to be identical to the extracellular domain of the GHR.<sup>56</sup> In rodents GHBP is generated by alternative splicing, but in humans through proteolysis of preformed GHR by the tumor necrosis factor- $\alpha$ -converting enzyme (TACE) and subsequent shedding of its extracellular domain to the circulation.<sup>57</sup> Its circulating concentration is thus believed to reflect the overall GHR expression status. Approximately 50% of circulating 22-kDa GH is bound to GHBP which prolongs the half-life of GH and serves as a buffer of GH in the circulation and at different target tissues.<sup>58,59</sup> It has been suggested that GHBP entails both inhibitory and stimulatory effects in GH signaling but its complete role is still uncertain.<sup>60</sup>

It was initially believed that the trigger for the intracellular signaling was the dimerization of the GHR induced by the binding of the GH molecule to the receptor.<sup>61,62</sup> However, it has later been shown that GH rather binds to the two different binding sites of a pre-formed dimerized receptor, and thereby a rotation of one of the receptor subunits is induced.<sup>63,64</sup> This conformational change enables the intracellular Janus kinase 2 (JAK2) to be repositioned, exposing its enzymatic kinase domain and triggering an intracellular signaling cascade by phosphorylating a conserved tyrosine in the cytoplasmatic signal transducers and activator of transcription proteins (STATs), particularly STAT5b.<sup>65</sup> The activation of STATs enables them to form homo- and heterodimers that in turn can translocate to the cell nucleus and promote the transcription of several GH target genes involved in growth and metabolism.<sup>66,67</sup>



**Figure 6.** Intracellular signaling pathways of growth hormone

Source: Reprinted by permission from Springer Nature: *Nature Reviews Endocrinology*, The growth hormone receptor: mechanism of activation and clinical implications, Brooks AJ, Waters MJ, Copyright (2010).

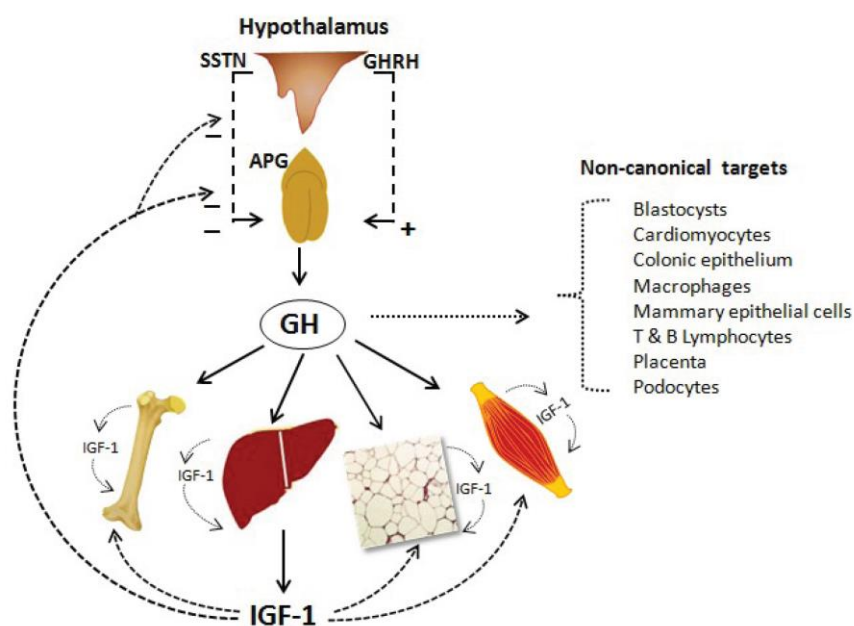
The binding of GH to the GHR triggers additional intracellular pathways illustrated above; the Src family kinases proto-oncogenes activate the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK)-pathway and the insulin receptor substrate (IRS) activates the phosphoinositide-3-kinase (PI3K)-AKT-mTOR-pathway, both highly involved in cell proliferation, metabolism and cell survival.<sup>68-70</sup>

### 2.2.5 GH/IGF-I-axis - the somatomedin hypothesis

The origin of the “somatomedin hypothesis” dates back to the landmark study by Salmon and Daughaday in 1957, in which they suggested that the effects of GH, or somatotropin as the hormone also has been referred to, was mediated through some other circulating factor.<sup>71</sup> This

factor was called “sulphation factor” based on its ability to stimulate cartilage sulphate uptake in both *in vivo* experiments of hypophysectomized rats and *in vitro* studies using costal cartilage of rats. They could demonstrate that the incorporation of radioactive sulphate in rat cartilages was reduced in hypophysectomized rats but could be restored by adding serum from normal rats or GH-treated hypophysectomized rats, in contrast to serum from non-GH-treated hypophysectomized rats or GH directly to the medium. Hence, the name “sulphation factor” for this unknown substance mediating the anabolic effects of GH in skeletal tissue.

The term *somatomedin* was later coined by Daughaday *et al* in 1972 to emphasize that this substance was increased by GH (*somatotropin*) and *mediated* the effects peripherally in both skeletal and non-skeletal tissue.<sup>72</sup> At that time, an understanding of the insulin-like properties of somatomedin, and its similarity to another described substance with non-suppressible insulin-like activity (NSILA) by Froesch and colleagues, was beginning to emerge.<sup>73,74</sup> Further investigations could establish that the newly determined peptides with NSILA, named insulin-like growth factor (IGFs) I and II, were identical to somatomedin type C and A and a universal nomenclature was proposed.<sup>75</sup> Later research findings have modified the original somatomedin hypothesis by illustrating a more complex relationship with both indirect effects of GH through IGF-I in an endocrine, paracrine and autocrine manner and direct effects on different tissues independent of IGF-I.<sup>76-78</sup>



**Figure 7.** The revised somatomedin hypothesis including both direct and indirect effects of growth hormone and non-canonical targets

*Note:* SSTN = Somatostatin, APG = Anterior Pituitary Gland, GHRH = Growth Hormone Releasing Hormone, GH = Growth Hormone, IGF-1 = Insulin-like growth factor I

*Source:* Reprinted from Kumar PA, Menon RK. New insights into growth hormone action on the macrophage: implications for non-growth-related actions of growth hormone. *OA Biochemistry* 2013 Oct 20;1(2):15.

## 2.2.6 Insulin-like growth factors

The peptides IGF-I and IGF-II are highly evolutionary conserved proteins of 70 and 67 amino acids each, with significant structural homology to proinsulin and crucial for regulating growth and metabolism in many cell types.<sup>79</sup> There are two known IGF receptors but both IGF-I and IGF-II bind primarily to the type 1 receptor (IGF1R) which has a sequence similarity varying between 41% to 84% to the insulin receptor, depending on the domain (the highest in the enzymatically active kinase domain).<sup>80,81</sup>

The majority of IGF-I in the circulation (up to 75%) is produced by the liver but virtually all tissues in the body can produce IGF-I, illustrating its important autocrine/paracrine capacity in addition to its endocrine properties.<sup>77,82</sup> IGF-II is in general considered to primarily be of importance for prenatal growth, illustrated by IGF-II-gene knockout mice models<sup>83,84</sup> and the genetic/epigenetic alterations in IGF-II-related human disorders such as Silver-Russell syndrome<sup>85</sup> or Beckwith-Wiedemann syndrome<sup>86</sup>. However, recent attention to its postnatal metabolic actions and links to obesity, type 2 diabetes and cardiovascular disease is also emerging.<sup>87-89</sup>

In the circulation and local tissue, the majority of the IGF molecules are bound to one of its six known binding proteins (IGFBP1-6) that can prevent the degradation of IGFs, modulate its action, facilitate transport and, to some extent, independently affect cell migration and proliferation.<sup>90-92</sup> The most abundant of the IGFBPs, and with the highest affinity for IGF-I, is IGFBP3. Together with another protein also produced by the liver, the Acid Labile Subunit (ALS), IGF-I, IGFBP3 (or IGFBP5) can form a large 150-kDa ternary complex which further increases the half-life of IGF-I, contains it in the circulation and prevents undesirable metabolic effects, such as hypoglycemia through binding to the insulin receptor.<sup>79,93</sup> All these three proteins (IGF-I, IGFBP3 and ALS) are central for human growth and under the stimulatory control of GH secretion.<sup>94-96</sup>

The hepatic production of IGF-I is to a large extent under the control of GH secretion but is also affected by nutritional intake<sup>97</sup> and insulin levels in the vena porta circulation<sup>98</sup> in which insulin affects GH-induced IGF-I production by stimulation of hepatic GHR transcription and translocation of GHR to the hepatic cell surface.<sup>99</sup> IGF-I levels also naturally vary over different periods in life, with the highest levels during puberty, in which sex steroids stimulate both GH secretion, modulate its peripheral actions and, as a consequence, increases IGF-I synthesis.<sup>48</sup>

## 2.3 PHYSIOLOGICAL EFFECTS OF GROWTH HORMONE

GH comprises a multitude of physiological effects in the body throughout life, by direct effects through its receptor and by interacting with other hormones. The two main effects involve longitudinal growth and the metabolism of carbohydrates, proteins and lipids. These effects will briefly be discussed in the following sections.



### 2.3.1 Longitudinal growth

Human growth can be divided into four separate phases; the fetal, infancy, childhood and pubertal phase, during which different factors such as genetics, nutrition, somatic and psychological well-being as well as hormonal factors contribute to various degrees.<sup>100-103</sup>

In the fetal phase, maternal factors, such as maternal nutrition and health, uterine size and placental function, play an important role in addition to primarily the actions of IGF-I, IGF-II and insulin.<sup>104-106</sup> The effect of congenital GH deficiency on birth size is quite limited but IGF-I deficiency will markedly impair intrauterine growth in both humans and animals, as shown in rare clinical conditions and knock-out mouse models.<sup>107-110</sup> The importance of GH gradually becomes more central during the infancy phase and plays a key role throughout the childhood and pubertal phases.<sup>111-113</sup> Several other hormones and growth factors are also important during the different phases of human growth, such as thyroid hormone, adrenal androgens, sex steroids, C-natriuretic peptide and fibroblast growth factors.<sup>114-116</sup>

The longitudinal growth promoting effects of GH and IGF-I within the growth plate is believed to be achieved by several mechanisms: recruitment of progenitor cells, increased cell division in the resting and proliferative zone, increased chondrocyte cell volume in the hypertrophic zone and stimulation of endochondral ossification.<sup>117-122</sup> The net effect is bone elongation and the current predominate conception is that GH has both IGF-I-dependent and IGF-I-independent actions in promoting its effects on bone growth.<sup>123,124</sup>

### 2.3.2 Metabolic effects

Although GH has received its name for its growth promoting effects, it is also highly involved in the body's metabolism of lipids, carbohydrates and proteins.<sup>125</sup> It executes these effects in various ways in different tissues, both directly and indirectly through interaction with mainly IGF-I and insulin.<sup>126,127</sup> From an evolutionary perspective, GH has an important role in regulating what energy fuel is used at different nutritional states, steering it away from carbohydrate and protein usage towards predominantly lipid usage. In short, GH promotes nitrogen retention, enhancing protein synthesis, in periods of food intake and alters the energy substrate from glucose and protein utilization to lipolysis in periods of fasting.<sup>128</sup>

#### 2.3.2.1 Growth hormone and lipid metabolism

GH has a prominent lipolytic effect and a single dose of exogenous GH will distinctly increase the amount of free-fatty acids (FFA) and ketone bodies in the circulation.<sup>129</sup> GH stimulates lipolysis in the adipose tissue primarily through stimulating the activity of hormone-sensitive lipase (HSL).<sup>126,130</sup> However, the effect on lipoprotein lipase (LPL) has



been shown to be either suppressive or non-existent suggesting that GH plays a minimal role in the lipid uptake of the adipose tissue.<sup>131</sup> In contrast, LPL expression is upregulated by GH in skeletal muscle which induces uptake of FFA and thus promotes lipid utilization as fuel in the muscle.<sup>132</sup> The same mechanism is also seen in the liver where GH also promotes uptake of FFA by stimulating hepatic lipase (HL) activity.<sup>133,134</sup>

#### *2.3.2.2 Growth hormone and protein metabolism*

The overall effect of GH on protein metabolism is anabolic both by its effects on protein synthesis and degradation but also by its effects on increasing the amount of alternative substrates for gluconeogenesis and thus diminishing the need of proteolysis.<sup>135</sup> In basal and periprandial states, most studies suggest a modest anabolic action of GH on protein metabolism, in contrast to states of stress and fasting where it has a more clear protein-preserving ability.<sup>127</sup> However, the main effects on protein synthesis and inhibition of muscle protein breakdown seem to be primarily mediated through IGF-I and insulin.<sup>136</sup>

#### *2.3.2.3 Growth hormone and carbohydrate metabolism*

Already in the initial descriptions of acromegaly, the connection of GH to carbohydrate metabolism was noted, as these patients often developed diabetes.<sup>3,137</sup> However, the most important contributions to clarify the metabolic effects of GH on carbohydrates were carried out by Dr. Bernardo Houssay and his team in the 1920s to 1930s, where they in hypophysectomized dogs observed fasting intolerance with severe hypoglycemia, high sensitivity to insulin and a diabetogenic effect of anterior pituitary extract injections in both normal and hypophysectomized dogs.<sup>138,139</sup> They could further show that the increased insulin resistance from administering extracts of the anterior lobe was also seen in animals without pancreas, gonads, thyroid or adrenal glands, indicating an independent mechanism of action rather than secondary effects on other hormonal systems.

Dr. Houssay was in 1947 awarded with the Nobel Prize in Physiology or Medicine for his ground-breaking work with the motivation: “for his discovery of the part played by the hormone of the anterior pituitary lobe in the metabolism of sugar”.<sup>140</sup> His findings were later confirmed in several famous studies by Frank Young, who induced diabetes in healthy dogs by injecting anterior lobe extracts and in 1937 stated that the “permanent diabetes produced in this manner differed from that of depancreatized dogs in that the pituitary dogs appear to be able to survive without insulin”.<sup>141</sup>

The effects on carbohydrate metabolism have later been found to be rather complex and presumably also linked to the dominant lipolytic effects of GH. It has in clinical studies been shown that infusion of GH decreases the uptake of glucose in skeletal muscle and decreases glucose oxidation probably due to an increase in lipid utilization in the muscles.<sup>142</sup> In the liver, GH stimulates hepatic glucose production by increasing glycogenolysis and to a lesser

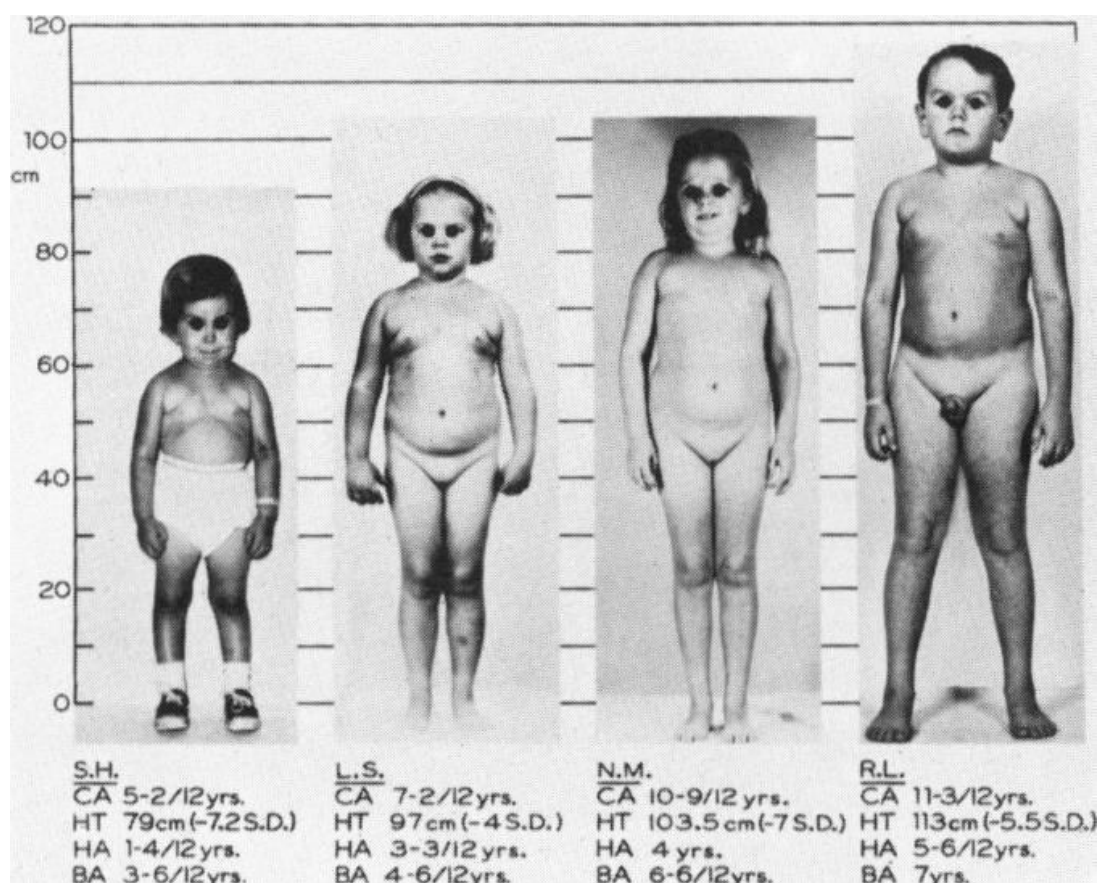
degree gluconeogenesis.<sup>126,143</sup> Lastly, GH also affects insulin secretion and signaling (see next section), further contributing to an overall increased insulin resistance and making it somewhat hard to disentangle the different contribution of direct effects on hepatic glucose production and indirect effects through increased hepatic and peripheral insulin resistance.

#### 2.3.2.4 *Growth hormone and insulin resistance*

Several mechanisms on how insulin resistance is induced by GH have been suggested, including both increased endogenous glucose production<sup>144,145</sup>, decreased glucose uptake in muscle tissue<sup>146</sup>, as well as effects on insulin signaling<sup>147-149</sup>. Thus, GH is believed to influence both hepatic and peripheral insulin sensitivity. Several of these effects may be due to the lipolytic effect of GH<sup>150,151</sup>, as mentioned above, which results in an increase of FFA and a shift in substrate usage for muscle tissue, as well as potential post-receptor effects on insulin signaling<sup>147,152</sup> and effects on  $\beta$ -cell insulin secretion<sup>153,154</sup>. However, GH has also been shown to have direct effects independent of the increase of FFA<sup>143</sup>, possibly through negative effects on the insulin receptor and the common post-receptor signaling pathway of insulin and GH, involving IRS and PI3K.<sup>148,149</sup> Even if there is a net effect on both lower glucose uptake in peripheral tissue as well as increased endogenous glucose production, the strict homeostatic control of serum glucose levels is maintained by higher levels of insulin secretion. One could speculate regarding a possible physiological advantage in creating a state of insulin resistance in order to not only attain the glucose regulating effects of insulin but also its anabolic effects in different phases of growth.

#### 2.3.2.5 *Metabolic profile of growth hormone deficient children*

As a consequence of the physiological effects of GH described above, severe deficiency of GH is associated with several metabolic features such as deranged body composition due to reduced lipolysis<sup>155-157</sup> and impaired protein anabolism<sup>127</sup> as well as decreased hepatic glucose production<sup>158</sup> and increased peripheral glucose uptake due to increased insulin sensitivity<sup>159</sup>. In the early works of Goodman *et al*, the clinical features were described as severely stunted patients with immature, doll-like facial appearance, with notably increased central fat deposits “giving a pudgy appearance”, small external genitalia and increased risk of hypoglycaemia.<sup>156</sup>



**Figure 8.** Clinical features of severe growth hormone deficiency

*Note:* CA = Chronological Age, HT = Height, HA = Height Age, BA = Bone Age.

*Source:* Reproduced with permission from Goodman HG et al. Growth and growth hormone. II. A comparison of isolated growth-hormone deficiency and multiple pituitary-hormone deficiencies in 35 patients with idiopathic hypopituitary dwarfism. *The New England Journal of Medicine* 1968;278:57-68. Copyright Massachusetts Medical Society.

Severe GHD is fortunately a rare condition and the majority of pediatric patients referred to endocrinologists due to short stature do not present with this phenotype. Nevertheless, quite few studies have investigated the metabolic features of short patients with less severe GHD. An exception to this, is however a study by Husbands *et al*, who reported that children with GHD, despite higher BMI, were more insulin sensitive than short children with normal GH secretion.<sup>159</sup> In addition, numerous studies examining the metabolic effects of GH treatment have demonstrated a decrease in insulin sensitivity, resulting in increased levels of insulin and C-peptide<sup>160-162</sup>, with only limited effects on fasting glucose and HbA1c in some studies<sup>163</sup> but also effects on these parameters in more recent investigations.<sup>164-168</sup>

A summary table of the major physiological effects of the GH/IGF-I-system in different tissues is presented below.

**Table 1.** Physiological effects of GH/IGF-I in different tissues

Organ	Main effect	Mechanism	Ref
Bone/growth plate	Promotes longitudinal bone growth	Stimulation of chondrocyte progenitor cells and the proliferation and differentiation of prechondrocytes through stimulation of systemic and local IGF-I synthesis as well as by IGF-independent mechanisms.	117,118,120-122
	Regulates bone remodeling	Stimulation of osteoblast proliferation and differentiation and, together with IGF-I, the recruitment and activity of osteoclasts.	169-172
Liver	Increases hepatic glucose production	Stimulation of glycogenolysis and (to some extent) gluconeogenesis, as well as increased hepatic insulin resistance.	143,144,173-175
	Decreases hepatic insulin sensitivity	Downregulation of insulin receptor and impairment of post-receptor insulin signaling.	147-149
	Increases uptake of triglycerides	Stimulation of hepatic lipase activity.	133,134
Adipose tissue	Increases lipolysis	Stimulation of hormone-sensitive lipase (predominantly in the visceral adipose tissue and to some degree in the subcutaneous adipose tissue).	126,130
	Decreases glucose uptake	Downregulation of glucose transporter-1 (GLUT-1).	126
Skeletal muscle	Decreases glucose utilization	Decreased glucose uptake and glucose oxidation.	129,146
	Increases lipid utilization	Upregulation of LPL and FOXO1 expression leading to increased lipid uptake and triglyceride synthesis.	131,132
	Impairs insulin signaling	Reduction of insulin receptor and IRS-1 levels with inhibition of the IRS/PI3K-pathway and induction of insulin resistance due to increased levels of FFA.	147-152
Pancreas	Increases insulin secretion	Stimulation of $\beta$ -cell proliferation and insulin secretion.	153,154

## 2.4 GROWTH HORMONE TREATMENT

### 2.4.1 The history of growth hormone treatment

Treatment with human GH has been in clinical use since the late 1950s and in a recombinant form since the mid-1980s.<sup>176</sup> However, attempts to treat human dwarfism with “purified” GH from bovine pituitaries started already in the 1930s, although with meager results.<sup>7</sup> The idea of GH as a species-specific hormone was postulated and the promising results of porcine and bovine insulin treatment in humans was not repeated with GH. The final proof of the “species specificity”-hypothesis was delivered by Ernst Knobil and Roy Greep in a famous study in Rhesus monkeys where they could demonstrate that primates would only respond to primate GH.<sup>177</sup> It has later been shown how this specificity is related to a single change in the amino acid chain of the GH protein which restricts the human GH receptor to only interact with GH of humans or other primates.<sup>178</sup>

In the end of the 1950s, the first purified preparation of human GH was described by Li and Papkoff<sup>179</sup> and the year after a simplified and more effective method was described by Raben<sup>180</sup>. In practice, the extracting and purifying process of GH from human cadaveric pituitaries was still a very costly and labor-intensive task in order to produce an effective amount of pituitary extract for treatment.<sup>181</sup> In the early days, approximately only 1 mg of hGH could be extracted from each pituitary gland and since that was roughly the same amount needed to treat one patient for one day, a total of 365 pituitary glands were needed for only one patient’s yearly treatment requirements. In 1963, an improved extraction and purification method using gel filtration was developed and published by the Swedish chemist Paul Roos *et al*<sup>182</sup> and the amount of hGH that could be extracted from a single pituitary gland continued to steadily increase during the 1970s due to further improvements in the purification process.<sup>181</sup> In the same decade, advances in identifying the biochemical structure of GH and the cloning of the GH gene for the first time in 1979 lay the groundwork for development of recombinant DNA-derived hGH.<sup>176</sup>

In 1985, the first reports of Creutzfeldt-Jakob Disease (CJD), in patients that had previously been treated with pituitary-derived GH, started to emerge and over the following years more than 200 cases related to contaminated cadaveric-derived treatment have been seen worldwide.<sup>183</sup> The majority of cases (119 of 226) have been reported from France and especially originating from a treatment period from December 1983 to July 1985.<sup>183</sup> However, around the same year, recombinant hGH (rhGH) became available for treatment and all use of pituitary-derived GH ceased. This was not only a major breakthrough in providing a “clean” substance to the patients with severe GHD but also creating a possibility of unlimited supply of GH, enabling the expansion of treatment indications to a larger number of patient groups.

## 2.4.2 Treatment indications and diagnostic challenges

From being an exclusive treatment given only to the most severe cases of GHD, when the supply was scarce, the advances in producing rhGH also made it possible to expand to less severe GHD patients as well as to other treatment indications. Current indications for GH treatment\* also include an increasing number of conditions in which childhood short stature is not primarily due to deficient endogenous GH secretion.<sup>184-186</sup> Examples of such conditions are idiopathic short stature, renal failure, Turner syndrome, or children born SGA. Conditions in which growth failure is a common feature but not assigned to defects in the GH/IGF-I-axis, thus expanding the indications from being a strict replacement therapy to also include a sort of “height enhancement therapy” for those patient groups.<sup>187</sup>

However, GHD is still the major treatment indication but this diagnose can also be quite challenging to determine.<sup>188</sup> Growth hormone is, as earlier described, secreted from the pituitary gland in a pulsatile manner with a very short half-life and often undetectable levels during large portions of the day. Thus, the diagnosis requires a combination of both clinical features, auxological patterns, information about parental height, investigation for other conditions leading to growth failure, possibly neuro-imaging as well as measurements of GH peak levels in response to different stimulation tests or spontaneous GH secretory profiles over 12 to 24 hours. Diagnosing the severe cases of GHD is often less challenging but those cases are rare and GHD can be considered as a continuum from total lack of secretion to less severe deficiency and borderline normal secretion.<sup>188</sup> Defining an adequate peak GH cut-off level for GHD in different stimulation tests has also been difficult due to large inter-assay variability in the assays measuring GH, different standardizations of the assays over time as well as considerable intrapersonal variability in the measurements.<sup>189-191</sup>

In the 1960s, GHD was defined as stimulated GH peak concentrations below 3 µg/L but over time the cut-off level gradually increased to 7 µg/L and later 10 µg/L.<sup>191</sup> Today, a stimulated GH-peak <7 µg/L is frequently used for defining GHD but a cut-off level of 10 µg/L is also often seen when GHD is distinguished from non-GHD forms of short stature, i.e. idiopathic short stature (ISS) or familial short stature (FSS).<sup>184,188</sup> The use of different standards for GH assay calibration have further complicated the issue. The introduction of recombinant calibration standards produced lower GH concentration values compared with the older pituitary-derived standards<sup>192-194</sup> and the conversion from different units (mU/L to µg/L) have added an additional source of error where large variations have been shown.<sup>195,196</sup>

The proposed continuum of GHD from total/severe deficiency to borderline/normal secretion is analogously illustrated in the responsiveness of GH treatment, where patients with severe GHD are highly responsive and patients with less severe GHD or ISS are not as responsive.<sup>197</sup> The difference of response could of course be due to several reasons; such as

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\* The term “GH treatment” will throughout this thesis be used synonymously with “recombinant human GH treatment” unless otherwise specified as pituitary-derived human GH treatment.

lack of compliance, difference in the degree of GHD as stated above, and variability in a certain patient's tissue responsiveness to GH.<sup>188</sup> With regards to the variability in response to a certain GH dose, a shift from fixed dosing to more individualized dosing is currently more common, adjusting the dose based on height and IGF-I response or alternatively using prediction model-based dosing in which different parameters, such as GH-peak in stimulation tests, growth velocity, IGF-I levels, difference in height standard deviation score (SDS) versus mid-parental height SDS, are taken into account.<sup>198-201</sup> However, to further complicate matters, maybe not only the individual variation in responsiveness to a certain level of GH might impact its various effects but the different GH secretion patterns with periods of peaks and lows might also contain different physiological effects. Studies in rats have indicated that the periodic pulsatile secretory pattern of GH may have implications on its different actions, where suggestively the peaks primarily affect growth and low periods in-between certain metabolic actions.<sup>202</sup> How this translates to humans is however still uncertain.

Lastly, the majority of patients that have received a GHD diagnosis in childhood based on abnormal stimulation test results will not have low GH-peak levels when re-tested after completion of growth.<sup>203-205</sup> This seems particularly true for those with idiopathic isolated GHD and with a normal magnetic resonance imaging of the pituitary gland. In one study, almost all (95%) childhood-onset idiopathic isolated GHD had normalized their stimulated GH peak response when retested.<sup>206</sup>

In summary, the challenges of diagnosing GHD remain largely unsolved and the cut-off levels are, from a physiological perspective, in a sense arbitrary and also burdened with issues of low reliability. Some patients with total lack of GH secretion will more easily be diagnosed but the majority of GHD patients are in-between total deficiency and normal levels. Furthermore, large patient groups with growth failure due to other reasons than deficiency of GH are treated, rendering the treatment with rhGH to more than just a replacement therapy and also introducing new ethical dimensions to the treatment decision.<sup>207</sup>

## **2.5 LONG-TERM SAFETY OF GROWTH HORMONE TREATMENT**

### **2.5.1 Effects of increased or decreased activity in the GH/IGF-I axis**

Based on the physiology of GH and its interaction with central mechanisms of growth and metabolism it seems clear that neither too much nor too little of GH is desirable in order to both develop normally and sustain a metabolism in balance. One way of better understanding the consequences of such disturbances and what potential long-term physiological effects they will have, is to study the “natural experiments” provided by Mother Nature, where activity in the GH/IGF-I axis is altered due to either acquired or congenital diseases.

A previously mentioned example of GH excess is acromegaly, where patients due to a GH-producing tumor of the pituitary gland have chronically increased and unregulated GH levels. This condition results not only in somatic overgrowth but also in impaired glucose tolerance with increased insulin resistance<sup>208</sup>, associated metabolic and cardiovascular morbidity<sup>209,210</sup> as well as an increased risk of colorectal tumors<sup>210,211</sup>. Furthermore, colorectal tumors seem to be related to high GH and IGF-I levels, as those with severe and uncontrolled acromegaly have higher risks compared to those with well-controlled disease.<sup>212</sup> Acromegalic patients have been reported to have a higher risk of cardiomyopathy, hypertension, arrhythmias, and type 2 diabetes mellitus (T2DM).<sup>210</sup> Studies have also shown that acromegalic patients have vascular alterations<sup>213,214</sup> and an increased risk to develop aneurysms<sup>215</sup>. In the last referenced study by Manara *et al*, it was also shown that the risk of developing aneurysms was positively associated with higher levels of GH at disease onset.

A contrasting example, with decreased activity in the GH/IGF-I axis, are patients suffering from Laron syndrome, who due to mutations in the GH receptor have impaired GH-signaling with very low levels of circulating IGF-I. Clinical features are similar to severe GHD with pronounced short stature, a certain facial phenotype and marked obesity.<sup>216</sup> However, despite their increased obesity, a recent study showed that subjects with Laron syndrome had lower incidence of T2DM, lower glucose and insulin concentrations and lower insulin resistance (measured by homeostasis model assessment of insulin resistance [HOMA-IR]).<sup>217</sup> This observation suggests an important role of intact GH/IGF-I signaling in the development of obesity-driven insulin resistance.



**Figure 9.** Ecuadorian cohort of patients with Laron syndrome together with their treating physician Dr. Jaime Guevara-Aguirre in 1988 (left) and 2009 (right)

Source: From Guevara-Aguirre, J., *et al.* (2011). "Growth Hormone Receptor Deficiency Is Associated with a Major Reduction in Pro-Aging Signaling, Cancer, and Diabetes in Humans." *Science Translational Medicine* 3(70). Reprinted with permission from AAAS.

Furthermore, studies from different Laron cohorts have also shown that their low activity in the GH/IGF-I axis seems to be protective of cancer development.<sup>218,219</sup> A study<sup>218</sup> from 2007



showed no malignancies in 222 patients with congenital IGF-I deficiency (169 patients with Laron syndrome and 53 with either GHRH receptor defects or GH gene deletion), with ages ranging from 3 to 78 years, and similar observations have been seen in two other studies<sup>219,220</sup> from 2011. These findings are also in line with a large systematic review published in The Lancet by Renehan *et al*, reporting an association between higher IGF-I and IGFBP-3 levels and increased risks of certain cancer types.<sup>221</sup>

Lastly, several animal studies have shown that mice with low GH/IGF-I activity are prone to live longer and have reduced incidence and/or delayed onset of neoplasms and other age-related pathologies.<sup>222-224</sup> Few studies have been done in humans, but a study by Milman *et al* showed that low IGF-I levels increased the predicted life expectancy in extremely long-lived females but not males.<sup>225</sup> For individuals with a previous history of cancer this association was seen for both males and females. Interestingly, this sex difference, with extended survival associated with lower IGF-I levels, has also been noted in other animal species.<sup>226</sup>

From normal physiology and the more extreme examples mentioned above, one can conclude that the old Greek saying “pan metron ariston” (translated as “everything in moderation”) seems appropriate also for GH/IGF-I activity. However, different types of morbidities might be expected at the different ends of the activity spectrum with tumorigenesis and insulin resistance in the higher end of GH/IGF-I activity and short stature, dyslipidemia and obesity in the lower end. With respect to the expansion of GH treatment indications beyond mere supplementation therapy to more of a “height enhancement therapy” in certain patient groups, where the activity of GH/IGF-I is not necessarily impaired, additional attention of morbidity in the higher end of the spectrum seems appropriate.

### **2.5.2 Surveillance of growth hormone treated patients**

The tragic events of Creutzfeldt-Jakob Disease in patients treated with pituitary-derived GH, described above, increased the awareness of the potential long-term risks and prompted the demand to closely monitor the patients treated with recombinant human GH. Several nationwide clinical treatment registries were initiated, such as the Swedish National Growth Hormone Registry, started in 1985, and in certain countries governmental agencies created mandatory registries of all patients started on rhGH treatment (e.g. Association Hypophyse in France). In addition, the pharmaceutical industry also initiated large registries of patients being treated with rhGH to closely monitor potential side effects and adverse events. Examples of such post-marketing surveillance registries are:

- KIGS – Kabi International Growth Study (Pfizer)
- KIMS – Kabi International Metabolic Study (Pfizer)
- NCGS – National Cooperative Growth Study (Genentech)
- GeNeSiS - The Genetics and NeuroEndocrinology of Short Stature International Study (Eli Lilly)
- NordiNet IOS – International Outcome Study (Novo Nordisk)

- PATRO Children – The Patients TReated with Omnitrope® (Sandoz)

Large number of patients have been included in these cohorts and several short-term side effects have been described, such as benign intracranial hypertension, epiphyseolysis, scoliosis, impaired glucose tolerance and pancreatitis.<sup>227-229</sup> Still, the overall short-term safety profile of rhGH has been considered favorable and certain early alarms of potential increased risk of leukemia<sup>230</sup> have not been seen in subsequent studies or in these post-marketing databases.<sup>227,231</sup>

However, several limitations of these post-marketing studies have been pointed out, such as the reliance on voluntary reporting of treating physicians, incomplete enrollment and outcome ascertainment, potential conflicting interest with the hosting pharmaceutical companies that control the access, analysis and release of data as well as lack of suitable comparison and control groups.<sup>187,229,232</sup> Moreover, the patients are only followed during their ongoing treatment which precludes the possibility to determine the long-term safety profile of the treatment and the possibility to discover rare events expected to manifest later in life.

These limitations and the continuous need to more properly address the issue of long-term safety in GH-treated children inspired the joint collaboration of eight European countries in creating a large meta-cohort study called SAGhE (Safety and Appropriateness of Growth hormone treatments in Europe).<sup>232</sup> The purpose was to analyze both efficacy on height, effects on quality of life as well as the long-term risks of future cancer incidence and mortality “independently of pharmaceutical companies”.<sup>232</sup>

### 2.5.3 Alarms from the French SAGhE cohort

On the 10<sup>th</sup> of December 2010, the European Medicines Agency (EMA) sent out a press release stating that the French Agency for the Safety of Health Products (AFSSAPS) had reported to them a suggested increased risk of mortality in patients treated during childhood with somatropin (GH).<sup>233</sup> The Food and Drug Administration (FDA) also published a press release shortly thereafter about the preliminary results from the French study but later pointed out, in April 2011, several methodological shortcomings which made it hard to draw clear conclusions from the data.<sup>234</sup> In the final report from EMA (EMA/H/A-107/1287) in February, 2012, emphasis was also put on the limitations of the French study but that the results should be regarded as a potential safety signal and further surveillance was needed.

These alarms stirred up a lot of emotions in the endocrine community and in 2012, Carel *et al* published their preliminary report showing an increased standardized mortality ratio (SMR) of 1.33 (95% CI; 1.08-1.64) of all-cause mortality in earlier treated subjects.<sup>235</sup> They noted an increased mortality risk with higher doses of rhGH and particularly the risk for bone tumor-related mortality (SMR 5.00, 95% CI; 1.01-14.61), diseases in the circulatory system (SMR 3.07, 95% CI; 1.40-5.83) and subarachnoid or intracerebral hemorrhage (SMR 6.66, 95% CI; 1.79-17.05). A brief report, published in the same issue of *The Journal of Clinical*

*Endocrinology & Metabolism*, from the SAGhE cohorts of Belgium, Sweden and The Netherlands was less alarming<sup>236</sup> and the robustness of the results have been questioned as stated above.

A study from 2016 by Albertsson-Wikland *et al* further elucidated this issue by showing that an observed increased SMR (1.43, 95% CI; 0.89-2.19) in Swedish childhood rhGH-treated patients with isolated growth failure (GHD, ISS or SGA without catch-up) normalized using a continuous hazard model also including birth characteristics (SMR 0.955, 95% CI; 0.591-1.460).<sup>237</sup> This illustrates the importance of trying to control for different confounding factors in these types of analyses. Nonetheless, the associations of these particular increased SMRs in the French cohort was disturbing, being both closely related to a central target tissue of GH (bone) and in unison with the previously reported link of increased activity of the GH/IGF-I axis and increased risk of cancer<sup>238,239</sup> as well as cardiovascular and metabolic morbidity<sup>240,241</sup>.

#### **2.5.4 Growth hormone treatment and cardiovascular and metabolic morbidity**

From the studies of GH physiology, the acromegalic patients and the preliminary results of the French study above, concerns of long-term cardiovascular and metabolic morbidity seem warranted. However, the improvement of dyslipidemia, decrease of central adiposity and increase of lean body mass with GH treatment in GHD patients seem to point in the opposite direction. An argument of continued GH treatment in adulthood of these patients has also been made with intention of improving their cardiovascular risk profile.<sup>242</sup>

A meta-analysis of placebo-controlled studies in GHD patients has indeed shown beneficial short-term (up to 1.5 years) effects of adult GH replacement treatment regarding body composition, diastolic blood pressure, LDL and total cholesterol but also negative effects on glucose and insulin levels.<sup>243</sup> In contrast, a Dutch study from 2013, regarding the metabolic effects of long-term (>10 years) adult treatment with GH in GHD patients showed a significantly increased risk of developing the metabolic syndrome (mainly due to abdominal obesity, hypertriglyceridemia and hyperglycemia) which is strongly associated to cardiovascular morbidity.<sup>244</sup> These conflicting results illustrate the complexity of this issue with potentially different mechanisms of developing insulin resistance, and a non-favorable metabolic profile, at hand.

The risk of developing type 1 diabetes mellitus (T1DM) in GH-treated patients does not seem to be increased<sup>227,228</sup> but in a study published in *The Lancet* by Cutfield *et al*, evaluating more than 23,000 GH-treated children, a 6-fold increase of type 2 diabetes mellitus (T2DM) incidence compared to two other age-matched cohorts of untreated children were reported.<sup>228</sup> Still being a rare event, the high increase in incidence and the lack of resolution even after GH treatment had been discontinued called for close monitoring of this potential consequence. When T2DM occurs in children or adolescents more than 80% are obese at

diagnosis but in the GH-treated children developing T2DM, obesity was much rarer, further supporting the idea of potentially a different pathogenic mechanism.

A more recent large observational study investigating the incidence of T2DM and glucose metabolism in over 5,000 patients with adult-onset GHD treated with rhGH showed similar results.<sup>245</sup> An increased incidence risk ratio (IRR) of 6.02 ( $p < 0.0001$ ) of developing T2DM in the treated group compared to the age- and sex-specific reference incidence rates in Sweden was reported. The increased IRR was largest the first year of treatment (10.8) to gradually decrease to 1.9 after 8 years of treatment. However, GH dose was not associated with development of T2DM but several other known risk factors were significantly associated, such as BMI, age, and waist-to-hip-ratio. This gradual decrease in the elevated IRR, with longer duration of GH treatment, could have several possible explanations; either that patients with higher risk of developing T2DM had an accelerated disease onset by treatment initiation or that there was an increased surveillance of blood glucose levels in the beginning of treatment which detected those at risk. One also needs to bear in mind the gradual increase of T2DM among young individuals in the background population when comparing incidence rates in treated cohorts with population-based incidence rates.

In experimental studies, direct vascular effects of GH<sup>246</sup> and IGF-I<sup>247</sup> have been seen which together with observational studies, such as the French mortality study cited above, have raised concerns regarding the possible increased risk of cerebrovascular disease in GH-treated individuals. In a study by Poidivin *et al*<sup>248</sup> these concerns were addressed and they reported an association of cerebrovascular morbidity (predominantly of hemorrhagic stroke) and GH treatment in childhood for isolated GHD or childhood short stature. However, this study has been criticized on several points, such as the use of questionnaires with low response rates and risk of responder bias, inappropriate comparison groups, questionable methods for compensating lack of exhaustive outcome data, and lack of important information regarding known and strong confounders (birth data such as birth weight and birth length).<sup>249,250</sup>

The effect of GH treatment on cardiovascular risk factors is a complex topic with possibly improved body composition and dyslipidemia as a positive force on the one hand and impaired glucose tolerance and increased insulin resistance as a negative one on the other. Direct effects on vessels and organs, such as the heart, is less clear even if seen in acromegalic patients, and few studies have evaluated actual cardiovascular events in previously GH-treated patients.

### **2.5.5 Growth hormone treatment and cancer risks**

The vigilance of a potential risk of developing cancer when treated with GH has accompanied this treatment since the beginning, being a potent mitogenic and anti-apoptotic hormone and based on studies showing that an increased activity of the GH/IGF-I axis is linked to increased cancer risk.<sup>238,239,251</sup> The link between GH and breast cancer has in particular been demonstrated in both epidemiological and clinical studies in which GH and its downstream

signaling molecules affect the different stages of tumor development and progression.<sup>252,253</sup> In animal studies of spontaneous dwarf rats (*Gh<sup>dr/dr</sup>*) which lack GH, administration of the cancerogenic substance *N*-methyl-*N*-nitrosourea did not induce mammary tumors but in GH-treated *Gh<sup>dr/dr</sup>*-rats tumors developed. Furthermore, after cessation of hormone replacement, nearly all developed tumors regressed completely.<sup>254</sup> Similar findings have also been described regarding prostate cancer development in an animal model of GHR-knockout mice (*Ghr<sup>-/-</sup>*) crossed with the Tag mouse prone to develop prostatic neoplasias.<sup>255</sup>

The increased cancer risk in acromegalic patients previously mentioned (section 2.5.1) and the reports from a small cohort study in the UK that showed increased mortality due to colorectal cancer and Hodgkin's lymphoma in patients previously treated with human pituitary-derived GH, have further spurred the attention of possible links between GH and cancer.<sup>256</sup> However, large post-marketing surveillance studies have not detected an increased risk in new primary cancers but raised risks of secondary malignancies in children treated with rhGH have been seen, which now also is listed in the U.S. labeling of all rhGH products.<sup>227</sup>

Despite the concerns of a possible carcinogenic effect of GH, long-term studies, properly powered to answer this question, are lacking. If GH treatment would have an effect on cancer development, one would expect this to occur after an extended time interval since cancer development generally includes a series of unfortunate events in the malignifying cell over time. Thus, long-term follow-up as well as large cohorts are needed to address this question. The SAGhE collaboration is an attempt to achieve this and the last project of this thesis covers the long-term cancer risk of GH treatment in childhood.

#### **2.5.6 Concluding remarks regarding the safety of childhood growth hormone treatment**

The issue of long-term safety of GH treatment in childhood is important to large groups of treated patients worldwide. Both biological and epidemiological studies warrant a concern for certain types of risks with this treatment, but addressing this issue is challenging for several reasons mentioned above. While large post-marketing studies have been reassuring regarding the short-term safety they also carry with them many limitations and there is still great uncertainty regarding the long-term safety of childhood GH treatment. This was the main motivating factor for the long-term studies regarding future cardiovascular and cancer risks included in this thesis with an attempt to address some of the methodological problems of earlier studies in order to bring more clarity to these important questions.



### **3 AIMS**

The overall aim of this thesis was to analyze the metabolic effects and long-term safety of recombinant human growth hormone (rhGH) treatment during childhood. In order to achieve this aim, the different projects cover a clinical trial with in-depth focus on metabolic effects of GH as well as large population-based cohort studies with focus on the long-term cardiovascular and cancer risks in childhood rhGH-treated patients.

The specific aims of the different projects were to:

#### **Study I:**

- Analyze if there are metabolic differences between short prepubertal individuals with stimulated GH peak levels in the lower normal range (7-14 µg/L) and healthy age- and sex-matched controls of normal height and weight.

#### **Study II:**

- Analyze how treatment with different doses of GH affects different metabolic parameters in the short prepubertal children of study I.

#### **Study III:**

- Analyze if patients being treated with rhGH during childhood due to GHD, SGA or ISS have an increased risk of cardiovascular morbidity and mortality compared to a randomly selected and matched comparison group from the general population.

#### **Study IV:**

- Analyze the risk of cancer morbidity and mortality in patients treated with rhGH during childhood compared to the general population.

## 4 METHODS

The methodology of the different studies included in this thesis will briefly be summarized in the following section. A more detailed description can be found in each separate article. The first two studies will be described jointly, being part of the same clinical trial, and study III and IV will be described separately.

### 4.1 STUDY POPULATION AND STUDY DESIGN

#### 4.1.1 Study I and II

##### 4.1.1.1 *Study design and setting*

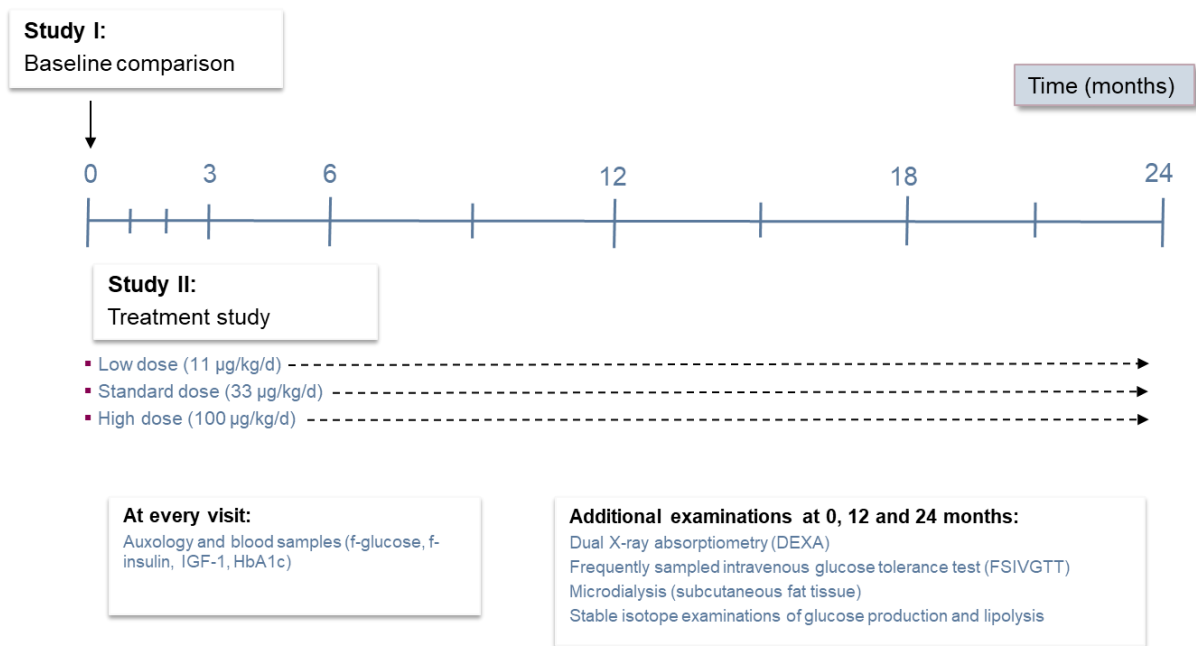
The first study of this thesis consisted of two parts; a baseline comparison between short prepubertal children with GH secretion in the lower normal range and a control group of healthy sex- and age-matched children of normal height and weight (Study I) and a randomized double-blinded two-year clinical trial of three different doses of rhGH treatment in the short children (Study II).

The study was an open multi-center study with four participating pediatric departments in Sweden, with the majority of included short children (32/35) from the Karolinska University Hospital in Stockholm. All investigations and in- and outpatient visits were performed at the Karolinska University Hospital in Stockholm. For the treatment study, the patients were randomized to three different doses of rhGH treatment; low (11 µg/kg/d), standard (33 µg/kg/d) or high (100 µg/kg/d) dose. The randomization was carried out by the pharmacy at the Karolinska University Hospital by a computerized sex-stratified block randomization to ensure a balanced allocation to each treatment arm and the double-blinded design.

##### 4.1.1.2 *Overview of Study I and II*

A more extensive in-patient examination of different metabolic parameters was carried out at 0, 12 and 24 months and the visits at 1, 2, 3, 6, 9, 15, 18 and 21 months were regular outpatient visits with auxological measurements and blood samples. An overview of the two studies and the follow-up visits is presented below.





**Figure 10.** Schematic overview of study I and II

#### 4.1.1.3 Study population

The study population consisted of 35 short children and 12 controls of normal height and weight with the following inclusion and exclusion criteria:

##### Inclusion criteria:

- Age: 7.0-9.9 years
- Height:  $<-2.5$  SDS (according to the Swedish growth reference<sup>257</sup>)
- Normal sitting height ( $\pm 2$  SDS, according to Gerver *et al*<sup>258</sup>)
- Term and normal birth weight for gestational age ( $\pm 2$  SDS according to Swedish birth reference<sup>259</sup>)
- Bone age  $<8.5$  years in girls and  $<10$  years in boys (assessed by RUS/TW2 – radius/ulna/short bones by Tanner-Whitehouse 2<sup>nd</sup> edition)
- Normal karyotype (girls)
- Prepubertal status (Tanner stage: B1, PH1 in girls and G1, PH1 in boys as well as testicle volume  $<4$  ml)
- GH peak value of 7-14 µg/L in an arginine and insulin tolerance test (AITT)

##### Exclusion criteria:

- Any syndrome or recognized disease that could compromise height
- Any ongoing medical treatment (except for well-controlled hypothyroidism)

The control children had to be healthy without any chronic disease or medication and of normal height and weight ( $\pm 1$  SDS according to the Swedish growth reference).

#### *4.1.1.4 Blood samples and metabolic examinations*

The investigations included blood samples of fasting insulin, fasting glucose, HbA1c, and IGF-I. Calculated values of insulin resistance using the homeostasis model assessment (HOMA-IR) was used both by traditional calculation ( $[\text{f-insulin} \times \text{f-glucose}]/22.5$ )<sup>260</sup> and through the up-dated computerized calculator (HOMA2-IR)<sup>261</sup>. The HOMA-indices have been shown to correlate well to the hyperinsulinemic-euglycemic clamp method, considered the gold standard of measuring insulin sensitivity.<sup>262</sup>

At the in-patient examination, calculation of insulin sensitivity measurements by frequently sampled intravenous glucose tolerance test (FSIVGTT), body composition examination by dual-energy x-ray absorptiometry (DEXA), analysis of markers of lipolysis in subcutaneous adipose tissue by microdialysis and whole body glucose production and lipolysis using stable isotope-labeled glucose and glycerol was performed. This in-depth characterization of the subjects' metabolic profiles, with focus on the carbohydrate and lipid metabolism, was the major methodological contribution to the research field of study I and II.

### **4.1.2 Study III**

#### *4.1.2.1 Study design and study population*

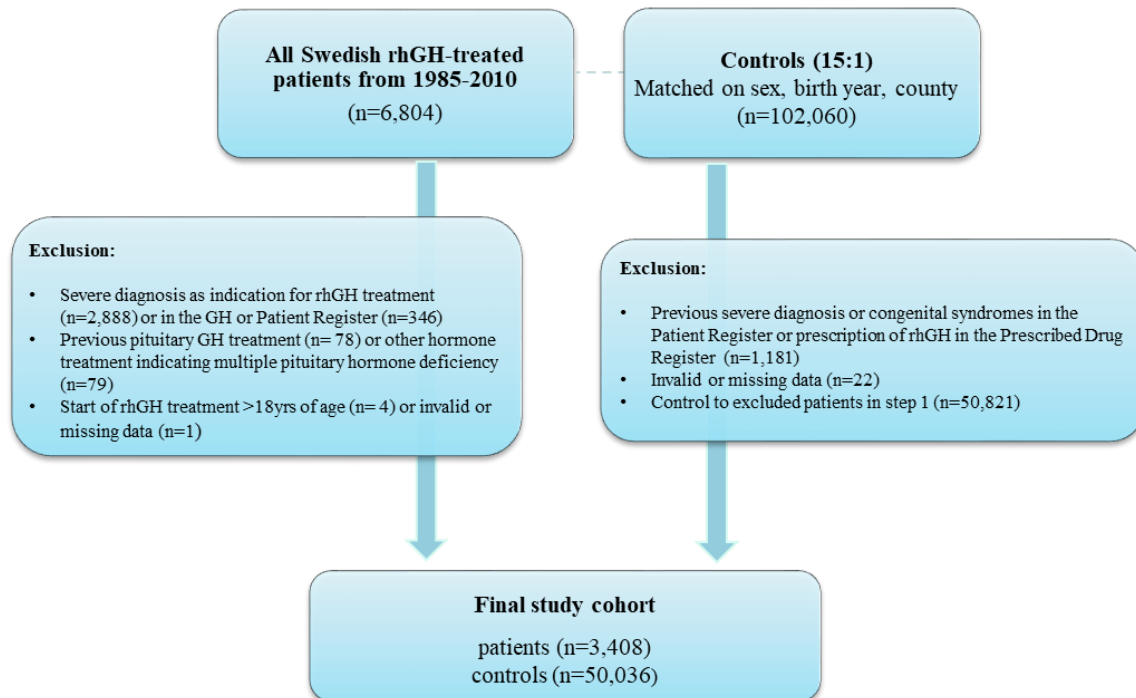
Study III was a population-based nation-wide cohort study of cardiovascular events in all Swedish rhGH-treated patients from the introduction of recombinant human GH in 1985 to the end of 2010. The rhGH-treated patients and their exposure data were collected from the Swedish National GH Registry for Children and clinical rhGH-trials during the same period, forming the joint GH-SAFETY cohort as described previously.<sup>237</sup>

A total of 6,804 patients were identified and for each patient 15 controls, matched on sex, birth year and county of residence, were randomly selected by Statistics Sweden (SCB) from the Swedish Total Population Register. Information regarding the parents of the patients and controls was also gathered from the Multi-Generation Register, creating a complete dataset of over 320,000 individuals.

After exclusion of individuals with previous severe diseases or syndromes, the final study population consisted of 3,408 patients and 50,036 controls. Examples of severe diagnoses, leading to exclusion, were previous malignant neoplasms, certain benign tumors and hematological diseases, endocrine disorders such as diabetes mellitus, congenital syndromic and metabolic disorders, chronic inflammatory diseases such as Crohn's disease, ulcerative

colitis, juvenile rheumatic arthritis, chronic renal diseases, chromosomal anomalies, and more (see Supplementary Appendix Table S1 of paper III for a complete list).

A summarized flowchart of the study inclusion and exclusion is presented below:



**Figure 11.** Flowchart of study inclusion and exclusion in study III

#### 4.1.2.2 Study outcomes and independent variables

The primary outcome of the study was the first cardiovascular event after the start of the follow-up, which equaled the starting date of rhGH treatment in the patient group and the corresponding age for the matched controls. The secondary outcome was to investigate the first occurrence of only severe cardiovascular events, which included aneurysms, ischemic heart disease, cardiomyopathy, heart failure and cerebrovascular disease.

Outcome data were obtained from the Swedish National Patient Register and from the Cause of Death Register. A cardiovascular event was defined according to the ICD codes of the 8<sup>th</sup>-10<sup>th</sup> revision of the International Classification of Diseases. A complete list of ICD codes used to define the outcome is presented in the Supplement Appendix (Tables S2) of paper III. Data on several potential confounding factors were additionally collected from different health- and population-based registries in order to adjust the analyses for these covariates.

No earlier study on long-term safety of rhGH treatment has to this extent included information on possible confounders, such as birth characteristics and socioeconomic status. The additional gathering of height data for the control group, from many different available sources, furthermore enabled us to adjust our analyses for height, that could be a proxy for many underlying factors connected to the risk for the outcome. By adjusting for both birth

characteristics, socioeconomic status and height and matching controls on sex, age and geographical county, we thus tried, as far as possible, to isolate the effect of earlier rhGH treatment on the studied outcomes.

**Table 2.** Summary of all variables and their sources in study III

Category	Variable	Source
<b>Outcome</b>	Cardiovascular events	The Swedish National Patient Register
	Cardiovascular death	The Swedish Cause of Death Register
<b>Exposure</b>	Treatment indication	The Swedish National GH Registry for Children and clinical rhGH-trials
	Treatment duration	
	Mean rhGH-dose	
	Cumulative rhGH-dose	
	Adult rhGH treatment	The Swedish Prescribed Drug Register
<b>Birth characteristics</b>	Gestational age	The Swedish Medical Birth Register
	Birth weight	
	Birth length	
<b>Socioeconomic status</b>	Parental educational level	The Swedish Register of Education
	Family income level	The Swedish Income and Taxation Register
<b>Individual factors</b>	Random selection of matched controls	The Swedish Total Population Register
	Link parent-child	Multi-Generation Register
	Height of patients at study start	The Swedish National GH Registry for Children and clinical rhGH-trials
	Height of controls at study start*	The Swedish Passport Register
		The Swedish Military Conscription Register
		The Swedish Medical Birth Register

\* Height data for the controls were gathered from several sources and height at study start was estimated using a mixed-effects model from all height measurements (for details, see Supplementary Appendix of paper III).

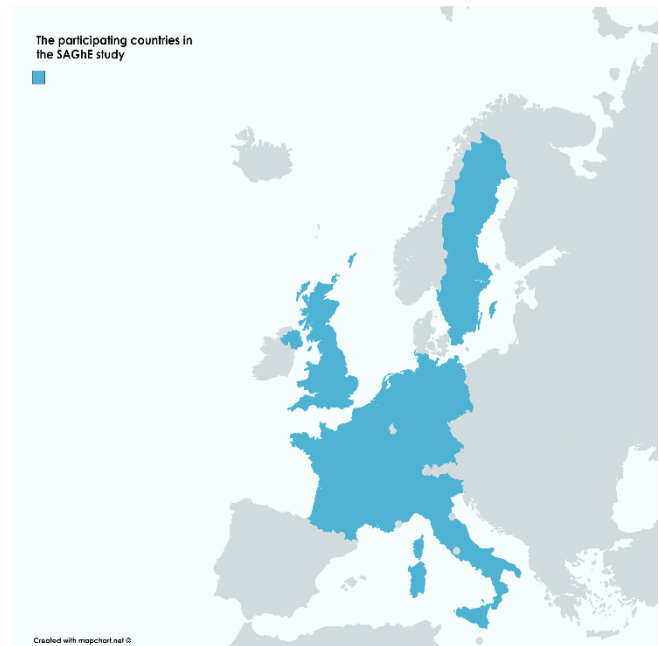
### 4.1.3 Study IV

#### 4.1.3.1 Study design and study population

Paper IV of the thesis is part of a joint collaboration between eight European countries, the Safety and Appropriateness of Growth Hormone Treatments in Europe (SAGhE) study, in which a large meta-cohort of rhGH-treated children has been assembled to evaluate its long-term effects and safety. This study addresses the issue of cancer risk in previously rhGH-treated patients.

The eight participating countries in the SAGhE study are France, Belgium, The Netherlands, Germany, Italy, Switzerland, the United Kingdom and Sweden.

A total of approximately 24,000 patients were included for the cancer mortality analyses with almost 400,000 person-years of follow-up. For cancer incidence analyses, only the countries with a national cancer registration with high coverage rate was included (Belgium, The Netherlands, Sweden, Switzerland and the United Kingdom) and for the other countries only numbers of cancer were reported.



**Figure 12.** The participating countries in the SAGhE study

#### 4.1.3.2 Risk group stratification, study outcomes and independent variables

Since the initial diagnosis leading to rhGH treatment is expected to be highly related to the outcome, all analyses were stratified based on a risk group classification connected to the underlying treatment indication.<sup>232</sup> In addition, certain diagnoses leading to rhGH treatment in which cancer risks are greatly increased, such as Type 1 neurofibromatosis, retinoblastoma, solid organ transplantation, and chromosomal syndromes (e.g. Fanconi or Bloom syndrome), were excluded. The risk group classification of a high, intermediate and low risk group corresponded largely to the arrangement of the patients into three overall groups; patients with an initial cancer diagnosis, a non-cancer diagnosis or only isolated growth failure (isolated GHD, ISS or SGA). All classification and exclusion were performed prior to any collection of outcome data or analysis.

The cancer mortality and incidence data were gathered from national population-based registries in Belgium, The Netherlands, Sweden and the United Kingdom, and by a multitude of sources, such as region-specific cancer registries, questionnaires, hospital discharge and

insurance records, for the other four countries.<sup>232</sup> Demographic and exposure data were mainly gathered from national population registries and from national population-based clinical treatment registries.

Several challenges are unavoidable when creating large meta-cohorts from several countries with different included sources and this complexity in the data needs to be considered when analyzing the aggregated data. Creating a large cohort from several countries was, on the other hand, necessary to reach sufficient statistical power when analyzing such rare events as cancer incidence and mortality in young adults. Country-specific analyses were also performed to assess the degree of national differences between the included countries. By the joint effort of the SAGhE consortium, the largest cohort of rhGH-treated children with the longest follow-up for cancer incidence and mortality was created, making it a very important contribution to the long-term surveillance of previously rhGH-treated children.

#### **4.1.4 Statistical analyses**

##### *4.1.4.1 Study I and II*

For the baseline comparison of the short children and the sex- and age-matched controls (Study I) the main statistical test was a two-sample independent *t*-test for approximately normally distributed variables and Wilcoxon rank-sum test for non-normally distributed variables. Similarly, correlation analyses were performed with Pearson product-moment correlation (*r*) for approximately normally distributed variables and Spearman's rank correlation coefficient (*r<sub>s</sub>*) for non-normally distributed variables. In the subgroup analyses of multiple groups, either ANOVA or Kruskal-Wallis test was used. The subgroups of patients were defined by a GH peak level above or below 10 µg/L. Post-hoc analyses were performed with Tukey's honestly significant difference for parametric data and Dunn's test for non-parametric data.

For the treatment study (Study II) the main statistical method was a mixed-effects model to account for the dependence in the data due to repeated measures at different time points of each individual and to more efficiently handle missing values in a series of measurements compared with other methods, such as for example a repeated-measures ANOVA. A mixed-effects model can be seen as a hierarchical model where the data are clustered on different levels, both on an individual level and on a higher level of different treatment groups. The name mixed-effects derives from the combination of both a *fixed* effect (treatment assignment) and a *random* effect (individual variation within each treatment group).

##### *4.1.4.2 Study III*

In study III, the main objective was to analyze time from start of the study (treatment start or corresponding age for the matched controls) to the first recorded cardiovascular event. The

most commonly used statistical method for time-to-event analysis, or survival analysis as it is also called, is the Cox proportional-hazard regression model, and this was also the method used for this study.

The Cox model estimates the *hazard function* or *hazard rate*, i.e. the risk of an event (“failure”) to happen at a certain point in time per time unit. When comparing hazard rates in different groups we calculate a ratio between the rates, the *hazard ratio* (HR). In our study of comparing the hazard rates for patient versus controls, a HR greater than 1 means that the risk of an event during the specified time interval is greater in the patients (exposed group) than in the controls (non-exposed group). If less than 1, the hazard is higher in the controls, and if equal to 1, the hazard rates are equal between the two compared groups. The estimations can be adjusted for multiple covariates making it possible to better isolate the effects of rhGH treatment when analyzing the association of treatment exposure and cardiovascular outcomes.

Two different adjusted models were used in our study apart from presenting crude HRs; a *restricted model* only including sex, age and height at study start, and a *full model*, which in addition included gestational age, birth length, birth weight, socioeconomic status (parental education and income quintile). Thus, the full model represents the estimated HR that fully takes all these potential confounders into consideration. The Cox model is based on a proportional-hazard assumption. We tested it by Schoenfeld’s residuals and visual inspection of the estimated hazard functions and found no evidence against it.

Analyses of subgroups of patients, categories of treatment exposure (treatment duration, mean and cumulative dose) and a sensitivity analysis on a subset of patients and controls more similar in height and birth characteristics at study start were also performed. The dose-response analyses were performed with a lag-period of two years since the end of treatment to avoid the risk of reversed causality (protopathic bias) due to the potential association of treatment termination and the outcome or early manifestations of the outcome.

For the estimation of height at study start in the controls, a mixed-effects model based on multiple height measurements of each control was used. The estimated value was the best linear unbiased predictor from the model and the analysis was done only in the control children and separately by sex. Due to non-linearity of the relationship between mean height and age over time, age was introduced in the model by means of natural cubic splines. The determination of the number and placement of the spline knots were done in order to maximize the goodness of fit as measured by the likelihood and also evaluated through graphical representation.

Crude incidence rates of cardiovascular events during the follow-up time with 95% confidence intervals (CIs) were also calculated for each subgroup of explanatory variables and presented as events per 10,000 person-years. Finally, Fisher’s exact test was used to analyze different proportions of events within each subcategory of cardiovascular ICD-codes.

#### 4.1.4.3 Study IV

The main statistical analysis to evaluate cancer mortality and incidence risks in study IV was calculation of standardized mortality ratios (SMRs), standardized incidence ratios (SIRs), and trends of SMRs/SIRs over categorical variables (p-trends). A SMR or SIR is a ratio between the *observed* number of cancer deaths or cancer incident cases in a certain study population and the *expected* numbers based on age- and sex-specific rates in the reference population.

$$SMR/SIR = \frac{\text{Observed number of deaths/incident cases}}{\text{Expected number of deaths/incident cases}}$$

Person-years at risk for cancer mortality were calculated for each patient by sex, calendar-year, 5-year age group and country, from date of first rhGH treatment to whichever occurred first: death, loss of follow-up, or a fixed censoring date (separate for each country). For cancer incidence, the same method was used but also adding the date of cancer diagnosis as an additional failure event. In analogy to HRs, an SMR or SIR over 1 means that the observed numbers exceeds the expected numbers, indicating an increased risk in the study population compared with the reference population. Absolute excess risks (AERs) were calculated by subtracting the expected from the observed number of cases, divided by the person-years at risk and multiplying by 10,000.

#### 4.1.5 Ethical approvals

All studies in this thesis have been approved by the Regional Ethics Review Board in Stockholm (Dnr: 01-069, 2010/578-31/1, 2011/109-32-1, 2011/305-32 and 2014/1775-32) and the clinical study (study I and II) was conducted in accordance with the ethical principles of the Declaration of Helsinki.<sup>263</sup>



## 5 RESULTS

### 5.1 STUDY I AND II

#### 5.1.1 Comparison of metabolic parameters at baseline (Study I)

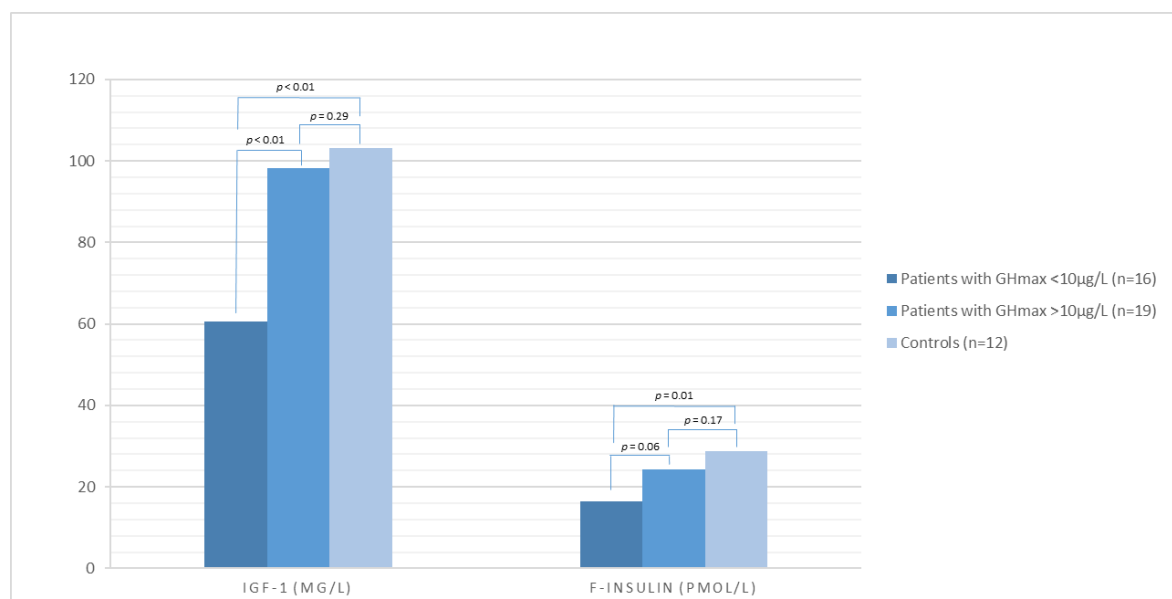
##### 5.1.1.1 Metabolic differences between short children and controls

In the overall comparison of the short children with GH peak levels in the lower normal range (GH<sub>max</sub> 7-14 µg/L) and the controls of normal height and weight, no significant differences in the investigated metabolic parameters were seen. Some tendencies could however be noted, where the patient group in comparison with the controls had lower fasting insulin (20.8 vs 28.8 pmol/L,  $p = 0.07$ ), IGF-I (83 vs 103 µg/L,  $p = 0.09$ ), HOMA indices (HOMA-IR; 0.75 vs 1.00  $p = 0.09$ , HOMA2-IR; 0.40 vs 0.55,  $p = 0.06$ ) and total fat mass (16.2 vs 19.6 %,  $p = 0.07$ ). No clear differences or tendencies were seen for the FSIVGTT, microdialysis or isotopic examinations.

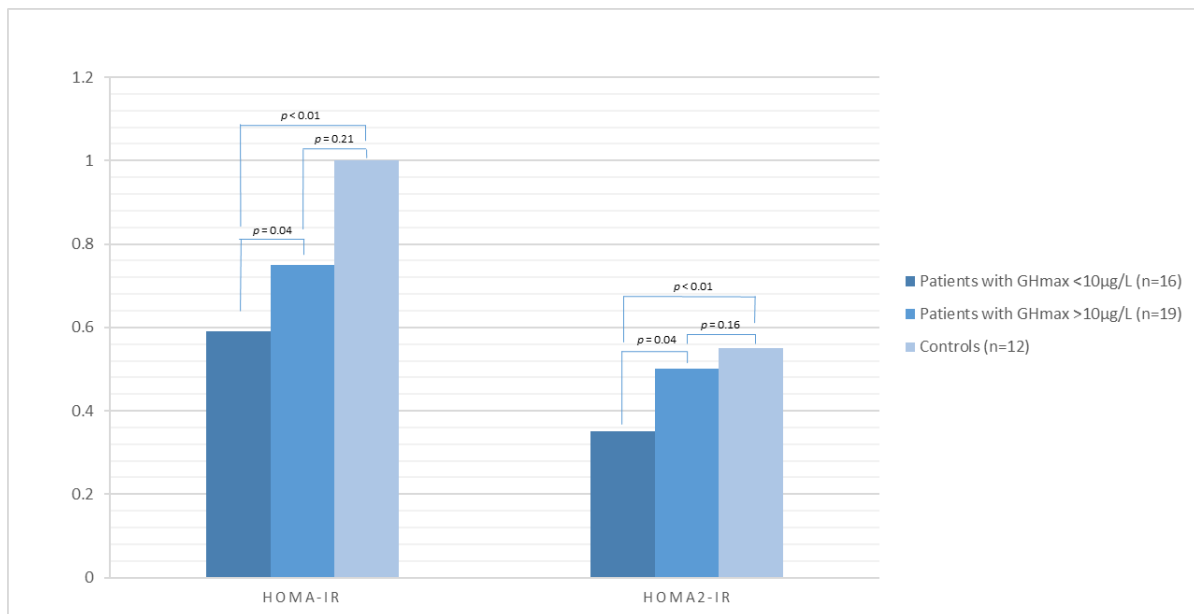
##### 5.1.1.2 Subgroup analyses based on GH peak levels above or below 10 µg/L

When dividing the patients into two subgroups based on their GH peak levels, using a common and “classical” cut-off for defining GHD or ISS (above or below 10 µg/L), several significant metabolic differences between the groups were seen. The three groups differed most clearly in measurements of IGF-I, fasting insulin and the HOMA indices. Post-hoc analyses showed that the subgroup of patients with the lowest GH peak levels (GH<sub>max</sub> < 10 µg/L) differed both from the patients with higher GH peak levels (GH<sub>max</sub> > 10 µg/L) and the controls. However, the subgroup of patients with GH<sub>max</sub> > 10 µg/L did not differ in these parameters or any other (except HbA1c) from the control children.

The comprehensive result table is found in paper I (table 3) and a selection of variables from the subgroup analyses, with p-values for the post-hoc analyses, is presented below:



**Figure 13.** Comparison of IGF-I and fasting insulin between the different groups



**Figure 14.** Comparison of HOMA-IR and HOMA2-IR between the different groups

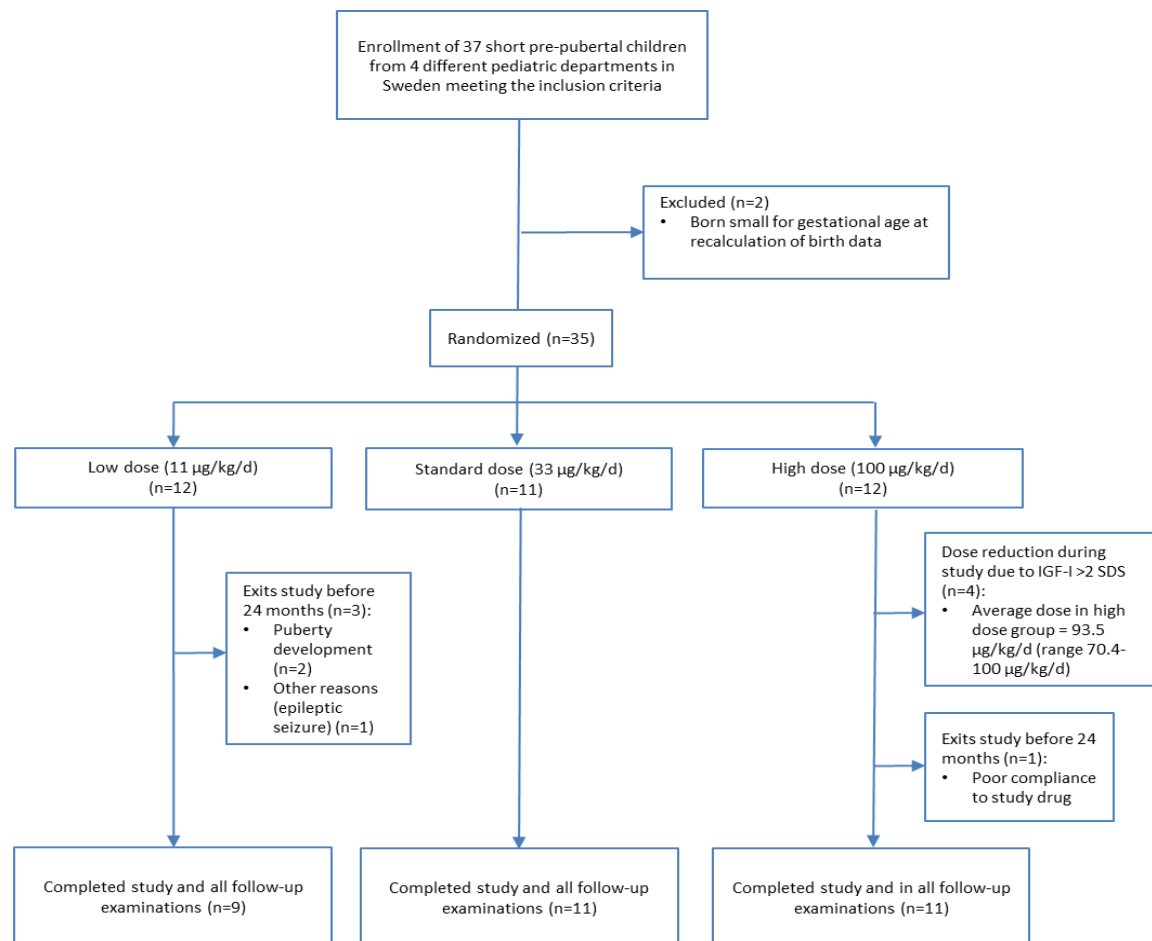
The subgroups of patients did not differ at all regarding body composition ( $p = 1.00$  on all three variables; abdominal fat mass, total fat mass and lean body mass). Neither did any of the subgroups differ significantly in body composition compared to the controls. Moreover, no differences were seen regarding FSIVGTT, microdialysis or the stable isotope examinations in the subgroup analyses.

## 5.1.2 Metabolic effects of different rhGH-doses (Study II)

### 5.1.2.1 Enrollment and completion of the treatment study

A total of 37 short prepubertal children were enrolled in the study with two participants excluded at a later stage due to being small for gestational age when recalculating their birth data. Of the 35 children that were randomized to the three different doses of GH, 31 completed the full two-year study and all follow-up examinations. Four children terminated the study before 24 months; three in the low dose group and one in the high dose group.

A flow chart of the study inclusion, reasons for exclusion or study termination and follow-up information is presented below.



**Figure 15.** Flow chart of study inclusion, exclusion and follow-up in Study II

#### 5.1.2.2 Metabolic differences between the three treatment groups at 12 and 24 months

At 12 months, differences between the treatment groups were seen regarding IGF-I, body composition, HOMA indices, and the FSIVGTT, in which particularly the high dose group had higher IGF-I levels, less total fat mass and decreased signs of insulin sensitivity. The standard dose group also showed lower insulin sensitivity (Si) in the FSIVGTT compared to the low dose group.

At the end of the study (24 months), similar differences as seen at 12 months were found, with the high dose group most clearly separated from the low and standard dose groups. In addition to the effects seen on HOMA indices earlier, the high dose group now also showed significantly higher fasting insulin levels and had over the study period a significant increase in fasting glucose (+0.6 mmol/l,  $p=0.004$ ) and HbA1c (+1.4 mmol/mol,  $p=0.046$ ).

The major differences in the metabolic outcomes are summarized below in table 3 and differences over time for a selection of variables in figure 16.

**Table 3.** Comparison of metabolic outcomes at 12 and 24 months

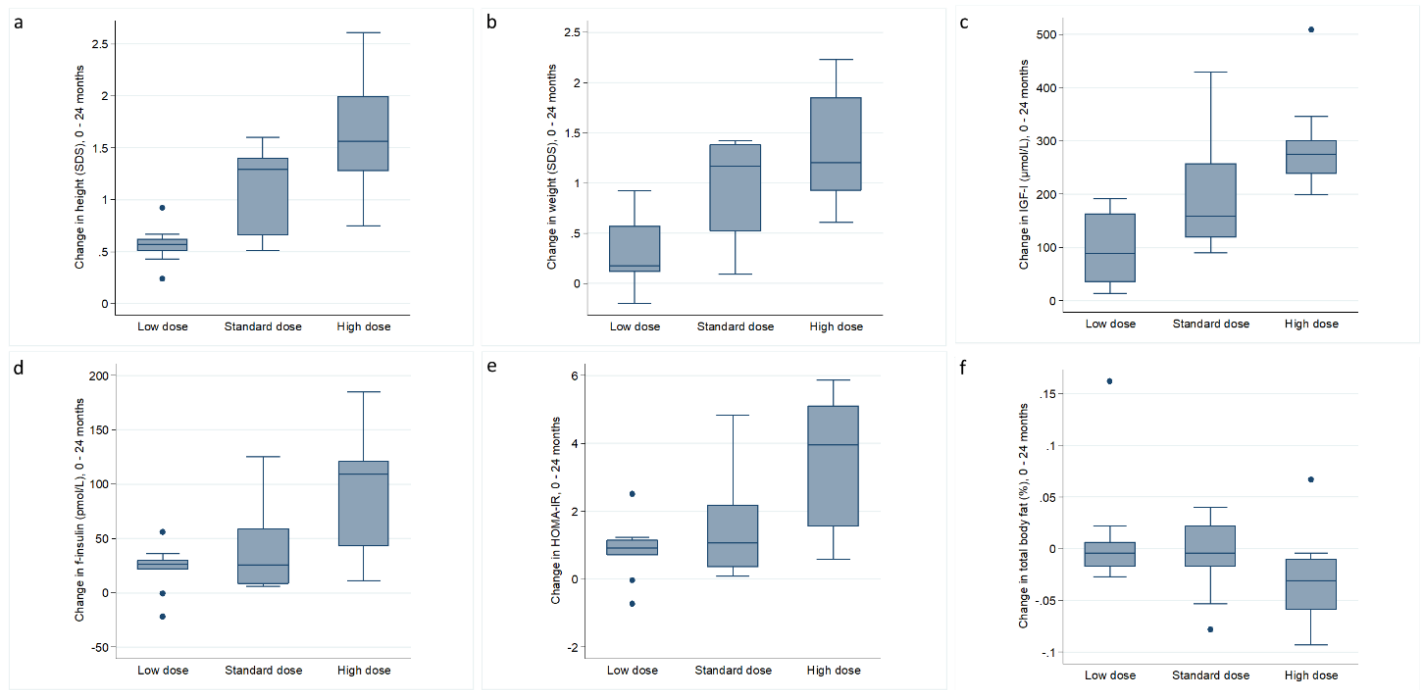
				<i>p</i> -values		
	Low dose (11µg/kg/d)	Standard dose (33µg/kg/d)	High dose (100µg/kg/d)	<i>Low vs Standard</i>	<i>Standard vs High</i>	<i>Low vs High</i>
<b>12 months</b>						
F-insulin (pmol/L)	52.5 (36.4)	43.5 (26.4)	74.7 (36.9)	N.S.	N.S.	N.S.
IGF-I (µg/L)	197 (98)	242 (62)	352 (121)	N.S.	<0.001	<0.001
HOMA-IR	1.99 (1.61)	1.51 (0.95)	2.77 (1.48)	N.S.	0.006	N.S.
HOMA2-IR	1.00 (0.70)	0.83 (0.49)	1.43 (0.72)	N.S.	0.012	N.S.
Si ([mU/l] <sup>-1</sup> x min <sup>-1</sup> )	10.1 (2.5)	6.4 (1.7)	5.4 (1.8)	0.025	N.S.	0.003
AIR (mUxL <sup>-1</sup> x min)	271 (203)	395 (300)	509 (264)	N.S.	N.S.	0.026
Tot. fat mass (%)	16.9 (6.2)	14.2 (6.1)	11.0 (4.8)	N.S.	N.S.	0.035
<b>24 months</b>						
F-insulin (pmol/L)	46.0 (22.9)	61.2 (35.8)	111.7 (52.9)	N.S.	<0.001	<0.001
IGF-I (µg/L)	220 (106)	291 (105)	402 (114)	0.007	0.002	<0.001
HOMA-IR	1.71 (0.93)	2.17 (1.38)	4.20 (1.94)	N.S.	<0.001	<0.001
HOMA2-IR	0.89 (0.44)	1.17 (0.69)	2.13 (0.96)	N.S.	<0.001	<0.001
Si ([mU/l] <sup>-1</sup> x min <sup>-1</sup> )	7.9 (2.0)	7.8 (2.9)	5.2 (2.5)	N.S.	N.S.	N.S.
AIR (mUxL <sup>-1</sup> x min)	348 (229)	418 (337)	667 (388)	N.S.	0.035	0.009
Total fat mass (%)	17.2 (9.6)	15.8 (6.3)	12.9 (6.3)	N.S.	N.S.	N.S.

Note: Mean (SD). IGF-I = insulin-like growth factor I, HOMA-IR/HOMA2-IR = homeostasis model assessment of insulin resistance, Si = (insulin) sensitivity index (measured by frequently sampled intravenous glucose tolerance test), AIR = acute insulin response (measured by frequently sampled intravenous glucose tolerance test), N.S. = non-significant.

### 5.1.2.3 Auxological and metabolic changes over the study period

Several auxological and metabolic changes did also occur over the study period within each treatment group. Changes from the start of the study to the end at 24 months of follow-up (delta [Δ] values, 0-24 months) showed a clear difference in both absolute values and within-group variation in Δ-values between the groups.

The standard and high dose groups had both larger Δ-values for height SDS, fasting insulin, HOMA-IR, as well as larger variation compared to the low dose group. In all the groups height SDS and weight SDS increased but most distinctly in the high and standard dose group. Effects on fasting insulin and HOMA-IR were most noticeable in the high dose group as well as the reduction of total body fat measured by DEXA. The Δ-values for a selection of variables are presented below in figure 16.



**Figure 16.** Changes in auxological and metabolic outcomes over the study period

The box-plots (a-f) show changes from baseline to the end of the study period ( $\Delta$ -values, 0-24 months) for the three treatment groups in different auxological and metabolic parameters.

## 5.2 STUDY III

### 5.2.1 Childhood rhGH treatment and long-term cardiovascular morbidity

#### 5.2.1.1 Characteristics of the study cohort and crude incidence rates of cardiovascular events

The final study population included 3,408 patients and 50,036 controls matched on age, sex and geographical region, with a total follow-up time of almost 800,000 person-years (pyrs). The mean follow-up time was 14.9 years and the mean age at study end was 25.1 years.

A total of 1,809 cardiovascular events were recorded during the follow-up, of which 167 were categorized as severe events. The crude incidence rates of a cardiovascular event were 25.7 events/10,000 pyrs among the patients and 22.6 events/10,000 pyrs among the controls. The rate was higher in female patients (31.2 events/10,000 pyrs) than in female controls (23.2 events/10,000 pyrs) but similar among male patients and controls (23.3 vs 22.3 events/10,000 pyrs). For the first severe cardiovascular event, the crude incidence rate was 3.48 events/10,000 pyrs in patients and 1.97 events/10,000 pyrs in controls.

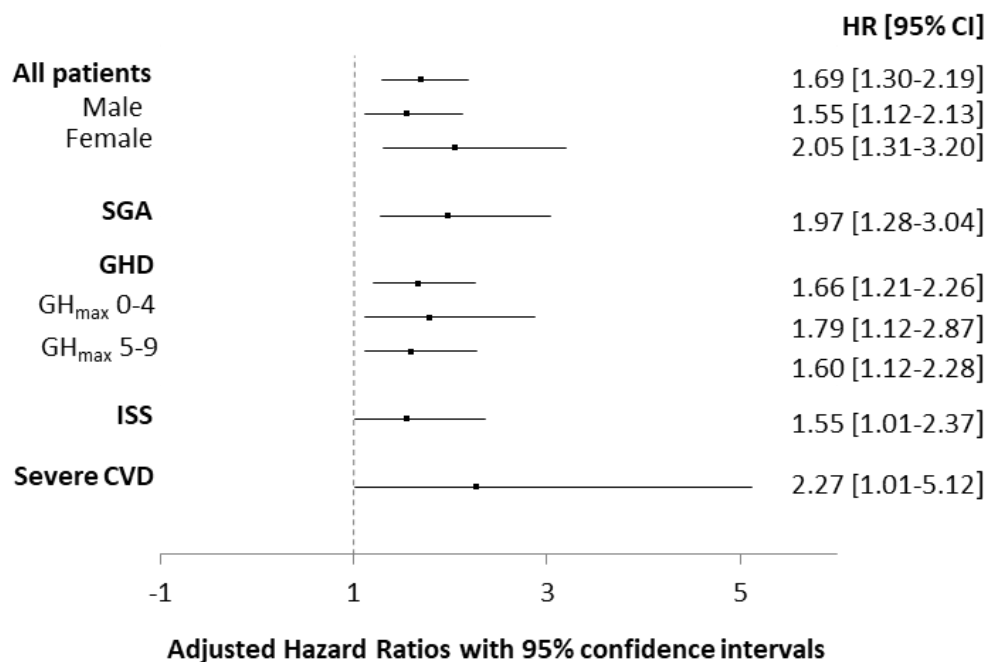
#### 5.2.1.2 Risk of overall and severe cardiovascular events

The main analysis of comparing time to the first overall cardiovascular event between the patient and the control group, showed a crude HR of 1.13 (95% CI: 0.95-1.36), which

increased in the restricted model (HR 1.58, 95% CI: 1.23-2.01) and furthermore in the fully adjusted model (HR 1.69, 95% CI: 1.30-2.19). This increased hazard was most clearly seen in the female patients (HR 2.05, 95% CI: 1.31-3.20). In the subgroup of patients, the increased HR was most evident in the SGA group (HR 1.97, 95% CI: 1.28-3.04) but also seen in GHD, defined as stimulated GH levels <10 µg/L, (HR 1.66, 95% CI: 1.21-2.26) and ISS (HR: 1.55, 95% CI: 1.01-2.37). For the subgroups of GHD patients (with stimulated GH levels of 0-4 µg/L and 5-9 µg/L) the HRs were 1.79 (95% CI: 1.12-2.87) and 1.60 (95% CI: 1.12-2.28), respectively.

For the first severe cardiovascular event, the crude HR was 1.75 (95% CI: 1.08-2.87) and also increased in the adjusted models (restricted model: HR 1.56, 95% CI: 0.73-3.34 and full model: HR 2.27, 95% CI: 1.01-5.12, respectively). The sensitivity analysis, only keeping controls more similar in height and birth characteristics at study start, demonstrated similar results as our overall analyses for both overall and severe cardiovascular events (see Supplement Appendix of paper III for details).

A forest plot of the different HRs in the main subgroups of patients is presented below for overall and severe cardiovascular events. A full list of all HRs for first overall and severe cardiovascular event is found in the tables 3 and 4 of paper III.



**Figure 17.** Forest plot of adjusted hazard ratios for first overall and severe cardiovascular event

The hazard ratios were adjusted for gestational age, birth length, birth weight, age at start, height at start, parental educational level, family income and sex (if not stratified for sex). HR = Hazard ratio, SGA = Small for gestational age, GHD = Growth hormone deficiency, GH<sub>max</sub> = Growth hormone peak level, ISS = Idiopathic short stature, CVD = cardiovascular disease.

### 5.2.1.3 Dose-response analyses

To further investigate any association with dose or duration, analyses by different categories of treatment duration, mean and cumulative dose were performed. The highest HRs were seen in the longest duration category ( $\geq 7$  years); 2.08 (95% CI: 1.35-3.20) and the highest cumulative dose category ( $\geq 4,500$  mg); 2.05 (95% CI: 1.18-3.55) with a significant increasing trend over the duration categories ( $p = 0.01$ ) but not over the cumulative dose categories ( $p = 0.24$ ). No association with higher mean dose and increased HR was seen.

**Table 4.** Dose-response analyses for overall cardiovascular events

	N	Adjusted* HRs [95% CI]	p-value
<b>Duration of treatment</b>			
0-2 years	925	1.05 [0.59-1.88]	0.87
3-6 years	1,522	1.58 [1.10-2.28]	0.01
$\geq 7$ years	961	2.08 [1.35-3.20]	<0.01
P trend		0.01	
<b>Mean GH-dose</b>			
0-29 $\mu\text{g/kg/d}$	402	1.76 [0.82-3.77]	0.15
30-39 $\mu\text{g/kg/d}$	2,383	1.64 [1.18-2.28]	<0.01
40-49 $\mu\text{g/kg/d}$	337	1.25 [0.60-2.59]	0.55
$\geq 50$ $\mu\text{g/kg/d}$	279	1.51 [0.79-2.91]	0.22
P trend		0.51	
<b>Cumulative dose</b>			
0-1,499 mg	1,015	1.34 [0.80-2.25]	0.26
1,500-2,999 mg	954	1.76 [1.16-2.68]	<0.01
3,000-4,499	902	1.36 [0.84-2.18]	0.21
$\geq 4,500$ mg	381	2.05 [1.18-3.55]	0.01
P trend		0.24	

\* Adjusted for gestational age, birth length, birth weight, age at start, height at start, parental educational level, family income and sex.

## 5.3 STUDY IV

### 5.3.1 Cancer risks after childhood rhGH treatment

#### 5.3.1.1 Characteristics of the study cohort and number of incident cancer cases and deaths

For the cancer mortality analyses, patients from all eight countries were included, totaling almost 400,000 patient-years, with an average of 16.5 years of follow-up per patient. For the cancer incidence analyses, only patients from Belgium, The Netherlands, Sweden, Switzerland and the United Kingdom were included, comprising a total of 154,371 patient-

years, with an average of 14.8 years per patient. A total of 251 cancer deaths and 138 incident cancer cases were recorded during the follow-up period.

#### *5.3.1.2 Cancer mortality by underlying diagnosis*

Overall cancer mortality was to a very large extent related to the underlying diagnosis leading to GH treatment. Patients treated with GH due to an initial cancer diagnosis had, as expected, a highly increased SMR for overall cancer mortality (SMR: 101.9, 95% CI: 89.6-116.0), especially for CNS tumors (SMR: 373.4, 95% CI: 318.6-437.5) and leukemia (SMR 45.5, 95% CI: 30.2-68.5), which contributed with 153 and 23, respectively, of the total of 230 cancer deaths. These specific cancer types were followed by bone tumors (n=8) and soft tissue tumors (n=5), which had SMRs of 35.5 (95% CI: 17.7-70.9) and 47.2 (95% CI: 19.7-113.4), respectively.

The remaining patients, with an initial non-cancer diagnosis, were further divided into patients with isolated growth failure (GHD, ISS or SGA), which contributed to more than half of the total cohort, and other non-cancer diagnoses (such as multiple pituitary hormone deficiency, Turner syndrome, and skeletal dysplasias). For isolated growth failure patients, the overall risk was not increased (SMR 0.8, 95% CI: 0.4-1.6) but the site-specific mortality for bone tumors was increased (SMR 3.1, 95% CI: 1.0-9.6). For other non-cancer diagnoses the overall cancer mortality risk was increased (SMR 2.2, 95% CI: 1.3-3.7) but no particular site-specific increased SMR was seen.

#### *5.3.1.3 Cancer incidence by underlying diagnosis*

As for cancer mortality, cancer incidence risk was highly influenced by underlying diagnosis. The overall SIR for those with an initial cancer diagnosis was 7.6 (95% CI: 6.1-9.6) and the most common location for a second primary malignancy was the central nervous system (n=23, SIR: 34.7, 95% CI: 23.1-52.2) followed by the thyroid gland (n=10, SIR: 32.2, 95% CI: 17.3-59.8), skin melanoma (n=5, SIR: 5.8, 95% CI: 2.4-13.9) and bone tumors (n=5, SIR: 17.2, 95% CI: 7.2-41.4).

For non-cancer diagnoses, overall SIR was 1.0 (95% CI: 0.6-1.4) in the isolated growth failure group, and 1.4 (95% CI: 1.1-1.9) for the other non-cancer diagnoses. No site-specific increased SIR was seen in the isolated growth failure patients but for the other non-cancer diagnoses, both bone (SIR: 4.1, 95% CI: 1.3-12.6) and bladder (SIR: 27.8, 95% CI: 7.0-111.3) malignancies were significantly increased, however based on few cases (3 and 2, respectively).

Both cancer mortality and incidence risks are summarized in table 5 below.



**Table 5.** Cancer mortality and incidence by underlying diagnosis

	N	SMR / SIR [95% CI]	p-value
<b>Cancer mortality</b>			
All initial diagnoses	251	13.7 [12.1-15.5]	<0.001
Initial cancer diagnosis	230	101.9 [89.6-116.0]	<0.001
Initial non-cancer diagnosis	21	1.3 [0.9-2.0]	N.S.
- Isolated growth failure (GHD, ISS, SGA)	8	0.8 [0.4-1.6]	N.S.
- Other non-cancer diagnoses	13	2.2 [1.3-3.7]	<0.05
<b>Cancer incidence</b>			
All initial diagnoses	138	2.2 [1.9-2.6]	<0.001
Initial cancer diagnosis	72	7.6 [6.1-9.6]	<0.001
Initial non-cancer diagnosis	66	1.2 [1.0-1.6]	N.S.
- Isolated growth failure	23	1.0 [0.6-1.4]	N.S.
- Other non-cancer diagnoses	42	1.4 [1.1-1.9]	<0.05

#### 5.3.1.4 Cancer mortality and incidence by demographic and treatment variables

In the comparison between males and females, similar SIRs were seen for both patients with an initial cancer diagnosis (M: 7.2, 95% CI: 5.0-10.3; F: 8.0, 95% CI: 5.9-10.8) and for those with an initial non-cancer diagnosis (M: 1.1, 95% CI: 0.7-1.7; F: 1.3, 95% CI: 1.0-1.7). In contrast, females had higher SMRs for both those with an initial cancer diagnosis (M: 90.2, 95% CI: 76.0-107.0; F: 123.1, 95% CI: 101.1-149.9) and those with an initial non-cancer diagnosis (M: 0.7, 95% CI: 0.3-1.5; F: 2.2, 95% CI: 1.3-3.7).

When analyzing time since start of treatment, overall diminishing risk for cancer mortality ( $p < 0.001$ ) but not cancer incidence ( $p = 0.13$ ) was seen in the total cohort with shorter duration since treatment cessation (with similar trends regarding treatment duration and cumulative GH dose). However, a time-lag analysis, censoring the first two years after the end of treatment, greatly diminished the association between higher SMR and shorter treatment duration, suggesting a possible reversed causality of treatment cessation due to cancer recurrence rather than cancer recurrence due to short treatment duration.

In patients with isolated growth failure, significant trends for higher cancer incidence were seen for increasing time since start of treatment ( $p = 0.02$ ) and duration of treatment ( $p = 0.02$ ) but not clearly for cumulative dose ( $p = 0.08$ ) and not at all for mean dose ( $p = 0.52$ ) or cancer mortality. In patients with an initial cancer diagnosis, a higher mean daily GH dose was associated with an overall higher SMR ( $p < 0.001$ ) but not higher SIR ( $p = 0.59$ ).

Analysis of site-specific cancer risks found diminishing risk of CNS tumor mortality in the initial cancer diagnosis group with longer-follow up ( $p < 0.001$ ) and increasing incidence of Hodgkin's lymphoma in initial non-cancer patients ( $p = 0.002$ ). No other clear trends were

seen regarding duration since treatment start and site-specific cancer mortality or incidence risks.

## 6 DISCUSSION

### 6.1 METABOLIC EFFECTS OF GH TREATMENT (STUDY I AND II)

In the first two studies, several metabolic effects connected to GH physiology were seen. One of the most noticeable findings was the strong association between GH and the effects on carbohydrate metabolism and especially on insulin sensitivity. This association was seen both in the comparison of short children with stimulated GH peak levels in the lower normal range and controls of normal height and weight (Study I) and in the assessment of various GH doses (Study II).

#### *Metabolic features of short prepubertal children*

In study I, differences between subgroups of patients (below or above GH<sub>max</sub> of 10 µg/L) were seen, indicating different metabolic phenotypes in the short children within the range of included GH peak levels. The subgroup of patients with GH<sub>max</sub> <10 µg/L showed lower IGF-I, fasting insulin and HOMA-IR/HOMA2-IR levels both compared to the controls and the short children with GH<sub>max</sub> >10 µg/L. However, no metabolic differences (except for HbA1c) were seen between the short children with GH<sub>max</sub> >10 µg/L and the control children. Moreover, no differences were seen in body composition between the subgroups of short children, indicating an association between lower fasting insulin and increased insulin sensitivity and low GH peak levels independent of differences in body composition. This observation is in line with the suggestion of alternative mechanisms on how GH contributes to decreased insulin sensitivity, affecting both central (hepatic) and peripheral insulin sensitivity discussed in sections 2.3.2.3-4.

Furthermore, this study also showed that the metabolic phenotype of short children with GH peak levels in the lower normal range differ substantially from the phenotype of patients with severe GHD, which have increased fat mass (especially abdominal fat mass) and BMI due to impaired lipolysis. Possibly, the level of GH secretion in our group of short children was sufficient to uphold a normal degree of lipolysis but at a low enough level to create a situation of increased insulin sensitivity, contributing to their phenotype of being both slim and short. Supporting this idea is also the findings from the stable isotopic examinations demonstrating similar levels of whole body glucose production and lipolytic activity between the groups.

Few earlier studies have investigated the metabolic characteristics of untreated short children in this range of stimulated GH peak levels (7-14 µg/L). However, an earlier study on short children with different levels of GH secretion have, similar to our results, shown that lower GH peak levels were associated with increased insulin sensitivity.<sup>159</sup> It was also shown that the group with lower GH peak levels had higher BMI, further supporting the notion of

mechanisms independent of body composition connected to the characteristics of increased insulin sensitivity.

### *Metabolic effects of different rhGH doses*

In study II, investigating the effect of different rhGH doses, a clear dose-dependent effect on fasting insulin levels and indices of insulin sensitivity was seen. Over time, all groups had increasing levels of fasting insulin, but the high dose group had significantly higher levels compared with both the standard and low dose group. Furthermore, insulin resistance measured by HOMA-IR and HOMA2-IR was distinctly increased in the high dose group compared to the standard and low dose groups.

Insulin sensitivity measured by frequently sampled intravenous glucose tolerance test, demonstrated decreasing insulin sensitivity in all groups over the study period but occurring earlier, already at 12 months, in the high and standard dose group compared to the low dose group. Possibly the higher amount of pubertal development in the low dose group could have contributed to reduce the difference between the groups at 24 months. Similarly, effects on body composition, demonstrating the lipolytic effects of GH, were also most clearly seen at 12 months, where the high dose group had lower total fat mass compared to the standard and low dose groups. Lastly, the high dose group had the largest increase in IGF-I and largest  $\Delta$  height SDS gain of the groups.

Similar results to ours have been reported in previous studies, showing increasing levels of fasting insulin and decreased insulin sensitivity during GH treatment in prepubertal children with or without GHD, but without any major impact on fasting glucose and glucose tolerance.<sup>160-163,264-266</sup> However, more recent studies on GHD patients have also reported effects on fasting glucose both associated with higher GH doses<sup>164</sup> and independent of dose<sup>165-168</sup>. In our study, the participants in the high dose group did also have a significant effect on fasting glucose and HbA1c and were, compared to recently published<sup>267</sup> pediatric references data, above the 95<sup>th</sup> percentile for fasting insulin, glucose and HOMA-IR but not HbA1c. The standard and low dose group were, on the other hand, within the normal ranges for these analyses even if demonstrating clearly increasing levels of fasting insulin and HOMA-IR over the study period.

## **6.2 CHILDHOOD GH TREATMENT AND LONG-TERM CARDIOVASCULAR RISKS (STUDY III)**

Study III, a population-based cohort study of Swedish rhGH-treated children from 1985 to 2010, showed that rhGH treatment due to GHD, SGA or ISS during childhood was associated with increased risk of cardiovascular events later in life. This was seen both for overall and severe cardiovascular outcomes, although the absolute risks were relatively low in both patients and controls, being rare events in a quite young cohort. The association was most

evident in female patients, patients treated due to SGA and in those with the longest duration of treatment and the highest cumulative dose. Additional analyses, comparing only patients and controls similar in height and birth characteristics at study start, or only those with documented treatment in adulthood (>18 years of age) from the Prescribed Drug Register, showed similar results as the main analyses.

The number of previous studies investigating long-term cardiovascular outcomes in rhGH-treated patients are very limited and most of them have primarily focused on cardiovascular mortality and reported unadjusted SMRs in adult-treated patients.<sup>268,269</sup> Furthermore, the included patients have a large variety of, and often severe, underlying conditions, making it difficult to draw definite conclusions regarding the impact of rhGH on the studied outcomes. Thus, in order to isolate the effect of rhGH, one must try to control for possible confounders and have a comparison group which ideally only differs in treatment exposure. In this study, a great deal of effort was put into this task, in order to, as far as possible, handle important confounders; such as birth characteristics, socio-economic factors and underlying (unmeasured) confounders associated with height and to filter out severe diagnoses in both patients and controls.

The hazard ratio for a cardiovascular event was approximately doubled for females overall and for the children treated due to SGA, although with quite wide confidence intervals, ranging from a third to a threefold increased risk. Earlier studies have also reported increased cardiovascular mortality risks in female versus male rhGH-treated adult patients but the underlying reason for this sex difference still remains unclear.<sup>268,269</sup> For mortality analyses, the low mortality risks in the female background population might explain increased SMRs, but this explanation seems insufficient for comparisons of morbidity.

The association of later cardiovascular diseases in children born SGA is well-known since the British epidemiologist David Barker formulated his hypothesis of fetal and infant origins of adult disease<sup>270</sup>, later confirmed by several studies<sup>271-273</sup>. In particular, rapid postnatal weight gain has been found to increase the risk of several cardiovascular outcomes in adult life.<sup>274-276</sup> However, the approved indication of GH treatment in SGA children is restricted to those who lack a catch-up growth, at least for height, in their first years of life. In theory, our subgroup of SGA children might thus have a lower baseline risk regarding cardiovascular diseases compared to other subgroups of SGA children with more rapid post-natal growth. Even so, we found a doubled risk of such outcomes when adjusting for birth characteristics, such as birth length, birth weight and gestational age. To further investigate this issue, one would need information on post-natal growth in both treated and untreated SGA children and preferably also identify other differences between the groups to further reduce potential residual confounding.

The argument of adult GH treatment in GHD patients is largely based on the metabolic effects of GH and particularly its favorable effects on lipid metabolism and body composition. Several studies on adult GH treatment have demonstrated such effects<sup>242,243,277</sup>

but others<sup>243-245</sup> have also reported negative effects, with increased insulin resistance and risk of type 2 diabetes mellitus, both findings consistent with the physiological effects of GH.

In our study, a sub-analysis of childhood rhGH-treated patients with treatment continuation after 18 years showed quite substantially higher HRs than our main analyses, arguing against an overall favorable effect on cardiovascular outcomes in our cohort (see Table S3, Supplementary Appendix of paper III for details). However, it is important to note that the studies cited above consist predominantly of patients with adult-onset GHD which, compared to childhood-onset GHD patients, differ greatly in etiology (mainly intracranial tumors or their treatment thereof disturbing the hypothalamic-pituitary axis) and level of deficiency (often a complete lack or very low levels of sustained GH secretion). In childhood-onset GHD, perhaps the severe forms with clearly deranged body composition and lipid metabolism could benefit from adult treatment, since such a phenotype would otherwise presumably lead to increased insulin resistance and subsequent cardiovascular morbidity. Thus, finding a balance of risk and benefit based on the underlying etiology and level of GH deficiency in the individual patient is most likely preferable in comparison to a strict general principle regarding adult treatment.

For severe cardiovascular outcomes, the patient group had approximately a doubled risk overall with, similarly as for the main analyses of overall cardiovascular events, higher HRs in the females compared to males. However, the absolute risks were even lower than for overall cardiovascular events, with few cases and wide confidence intervals. When comparing the proportions of specific severe cardiovascular outcomes, the patient group had a significantly higher amount of ischemic cardiac disease and cardiomyopathy. The link of GH excess and effects on the heart is well-known since cardiomyopathy with biventricular hypertrophy of the heart is one of the hallmark cardiac complications seen in acromegalic patients.<sup>278,279</sup> On the other hand, GHD is also associated with changes in heart morphology and function, with decreased cardiac size and cardiac output, opposite to the effects of GH excess.<sup>279</sup> Thus, avoiding both too high and too low levels of GH seems once again advisory. With regards to rhGH treatment, abiding to approved treatment indications and carefully monitoring effects of the treatment (IGF-I levels and growth response), is likely to be favorable in order to reduce such future cardiac morbidity risks.

### **6.3 CHILDHOOD GH TREATMENT AND CANCER RISKS (STUDY IV)**

Study IV, part of the SAGhE study, is a joint collaboration of eight European countries to create a large meta-cohort of childhood rhGH-treated patients with one of its objectives to investigate the long-term cancer risks in earlier treated patients. The major findings from the study was that the cancer mortality and incidence risks are highly associated with the underlying diagnosis for receiving rhGH treatment. The vast majority of cancer deaths and incident cases were a consequence of secondary malignancies in patients with a previous cancer diagnosis.

For the patients without a previous cancer diagnosis, a slight increase in cancer incidence was seen but not in cancer mortality. Further separating this group into subgroups of patients with isolated growth failure (GHD, SGA, and ISS) or other non-cancer diagnoses (such as multiple pituitary hormone deficiency, Turner syndrome, and skeletal dysplasias) showed that the former did not have an overall increased risk of cancer mortality or incidence but the latter did for both cancer mortality and incidence. In the patients with isolated growth failure, site-specific increased mortality risk of bone tumors was seen but no site-specific increased incidence risks. Even if significant, the number of deaths from bone tumors were few (n=3) creating wide confidence intervals for the three-fold increased SMR. For the other non-cancer diagnoses, no site-specific increased cancer mortality risk was seen, but increased site-specific incidence risks for bone and bladder malignancies were found.

The lack of large-scale long-term studies investigating cancer risks in previously rhGH-treated patients greatly motivated this study. The follow-up studies to date have either had short follow-up time<sup>227,280-282</sup> or small cohorts<sup>283,284</sup> and have largely been based on post-marketing databases, which rely on active reporting of adverse events by the treating physician<sup>227,280-282</sup>. Although many patient-years have been gathered in these databases, few conclusions of long-term safety can be drawn since the mean follow-up time per patient is only around four years. Considering that the development of malignancies often has quite a long lag period, an extensive follow-up time is needed to be able to detect any link to previous GH treatment. Most cancer types also occur in adulthood, and short follow-up time in still young patients would not provide any information on the risk of developing such malignancies later in life.

Even if overall reassuring results in patients with isolated growth failure, the increased cancer mortality seen for bone tumors is somewhat disturbing since one of the main targets of GH, with abundant expression of the GH receptor, are the chondrocytes and osteoblasts (see section 2.3.1). For the other non-cancer diagnoses, increased incidence but not mortality for bone tumors was also seen, as well as several cases in both the mortality and incident analyses for the patients with a previous cancer diagnosis. A general increased risk of secondary neoplasms in childhood cancer survivors has been reported earlier by Sklar *et al*, and of note was the occurrence of osteogenic sarcomas in leukemia and lymphoma survivors treated with GH.<sup>285</sup> An increased risk of CNS tumors, mostly meningiomas, was also reported in the same study and similar findings were reported by Bell *et al*, with osteogenic sarcoma being the second most common secondary neoplasm in cancer survivors.<sup>227</sup> Both meningiomas and osteogenic sarcomas have been shown to express receptors for GH and IGF-I and modulation of the GH/IGF-I axis can affect the growth of these tumors in vitro and in vivo.<sup>286,287</sup> However, regarding meningiomas, a separate report from the SAGhE cohort has shown that this finding is probably mainly due to cranial radiotherapy in childhood cancer survivors, with the role of GH treatment being more uncertain.<sup>288</sup>

Another finding for the group with initial non-cancer diagnoses, apart from isolated growth failure, was the increased incidence of bladder cancers of which only very scarce previous

data related to the GH/IGF-I-axis exist. In a case report from Japan, the recurrence of a superficial bladder tumor was described to be associated with periodically high levels of serum GH in an acromegaly patient.<sup>289</sup> Bladder cancer has also been described to overexpress the growth hormone genes *GH1* and *GH2* by reports from the Oncomine database, a cancer microarray database of the cancer transcriptome in a large variety of cancer types.<sup>290</sup> Moreover, the detection of GHRH receptors in a series of bladder cancers have led to experiments with both GHRH agonist and antagonist showing tumor reducing capacities in mice xenografted with human bladder tumor cell lines for both substances, possibly by downregulation of GHRH receptors in both the cancer cells and the pituitary gland.<sup>291,292</sup> A recent meta-analysis of cancer incidence in patients with acromegaly has also reported a slight, but increased, SIR for urinary tract cancers.<sup>293</sup> However, the significance of our finding in the patients without a previous cancer diagnosis remains uncertain until more supporting data exist.

A previous study on patients treated with human pituitary-derived GH has reported increased overall cancer mortality risks, particularly from colorectal cancer and Hodgkin's lymphoma.<sup>256</sup> Colorectal cancers have also been the main increased cancer type seen in acromegalic patients.<sup>294</sup> However, in our study, very few cases of colorectal cancer were seen (n=6) with a significant increased standardized incidence ratio only in patients with an initial cancer diagnosis, based on two cases. For Hodgkin's lymphoma, no overall increased risk was seen in any of the groups but for those with an initial non-cancer diagnosis, a highly significant increased incidence was seen with longer follow-up.

The analyses of treatment dose and duration did not find any significant effects of higher cumulative dose or longer duration of treatment in patients with no previous cancer diagnosis, arguing against an overall effect on cancer risks in these patient groups. For isolated growth failure separately, increasing cancer incidence but not mortality was seen with longer treatment duration and follow-up time but not clearly for cumulative dose and not at all for mean dose. In those with an initial cancer diagnosis, higher mean dose was associated with increased cancer mortality but not cancer incidence. The interpretation of this finding remains unclear with arguments both for and against a possible causal explanation and further data are needed to clarify if higher GH doses might affect cancer survival in patients with a previous cancer diagnosis.

## **6.4 STRENGTHS AND LIMITATIONS**

### **6.4.1 Study I and II**

The major strengths of study I and II, was the multitude of different metabolic investigations used to explore different metabolic parameters in this patient group of short children in comparison with controls of normal height, as well as the effects of different rhGH-doses over time. The in-depth examinations elucidated several key findings of the interplay between the GH/IGF-I-axis and other metabolic and hormonal systems, such as glucose metabolism



and insulin sensitivity. Not only were several differences between the groups and subgroups found, but also indications of the underlying mechanisms that created these differences. The group of short children was also strictly selected and examined in a consistent manner at the same research center which provided more robustness to the results and diminished the risk of introducing bias by different examiners and/or research centers. For study II, the design of having an external unit to handle both the block randomization process and the drug administration in unmarked packages could guarantee the blinding of dosing for both the patients, parents and study investigators.

The limitations of study I and II were the size, which led to insufficient statistical power in possibly reaching significance for many of the non-significant trends of study I and restricted further subgroup analyses in study II. This could also increase the risk of making Type II errors in assuming the null hypothesis to be true when it is not, and we should always remind ourselves that the absence of proof does not equal proof of absence. The overall recruitment process was more difficult than anticipated when designing the study and due to the amount of different examinations carried out, each included patient was also very labor-intensive. A further limitation was the lack of information on how many eligible children were asked and declined participation at the other sites apart from the main recruitment site at the Karolinska University Hospital. However, only three out of the 35 included patients came from other sites and this limitation is unlikely to have affected the overall results of the study. For study II, the loss of follow-up in four patients is a limitation, but with almost 90% of the included patients fulfilling the two-year follow-up and the lack of association with dose allocation in those who terminated the study prematurely, it seems unlikely to have introduced any bias to the results.

#### **6.4.2 Study III**

Study III is a population-based nation-wide cohort study with 25 years of follow-up, covering almost all Swedish rhGH-treated patients from the introduction of recombinant GH in 1985 to the end of 2010. Over 3,400 patients with GHD, SGA or ISS, and more than 50,000 controls were included in the analyses of possible cardiovascular risk in childhood rhGH-treated patients.

Through extensive linkage of data from numerous Swedish healthcare and population-based registries, using each patient's and control's unique personal identity number, we were able to address this question in a more profound and detailed way than has been previously done. The controls were randomly selected by Statistics Sweden from the general population and matched to each patient based on sex, age and geographical region and information not only regarding the patients and controls but also on the parents was gathered in order to adjust for socioeconomic factors within the family. Fifteen controls for each patient were selected and other important covariates for the adjusted analyses, such as birth characteristics (birth length, birth weight and gestational age), could also be obtained for the whole cohort. Outcome data

were retrieved from the Swedish National Patient Register and Swedish Cause of Death Register, which both have very high and nearly complete coverage rates, keeping the incompleteness of follow-up to a minimum. All outcome data were independently and prospectively collected within the registries, minimizing different types of information bias.

To further strengthen our analyses, we also gathered information on height for the control group from several sources, in order to adjust for this covariate and thereby also controlling for other height-associated confounding factors which are hard or impossible to measure. We could thus not only adjust for birth characteristics, age, sex, geographical and socioeconomic factors, but also for height at study inclusion, in our attempt to isolate the effects of GH treatment on the analyzed outcomes.

The major limitation of the study is the possibility of confounding by indication, where the underlying reason for starting GH treatment might also be linked to the risk of later cardiovascular events. We tried to handle such potential bias by the measures described above and also by performing dose-response analyses to investigate if treatment exposure was of relevance or not for the risk of future events. However, it is impossible to completely avoid the possibility of residual confounding, and the associations described should be seen in this light and not be viewed as evidence of causal relationships.

Another limitation concerns the fact that our cohort is still young and we only have data on long-term effects up to young adult ages. This limitation restricts us to infer about risks in later adulthood and makes further surveillance important for such questions to be answered. Being a young cohort also introduces issues of statistical power; even if we gathered a large cohort, the outcome we study is still quite uncommon in young individuals and therefore the number of events are low. This weakens the precision in our estimates and makes some of the stratified subgroup analyses underpowered. The continuous follow-up to older ages is thus also important for this aspect, since cardiovascular events will become more frequent in later adulthood.

#### **6.4.3 Study IV**

The collaboration of several countries to create a large cohort of previously rhGH-treated patients has been a major effort and has provided valuable information on such rare events as cancer incidence and mortality in a still quite young group of patients. Another strength of this study, apart from its size, is the extensive follow-up time, which is necessary to investigate events with an expected long lag period. The high completeness of the study, through gathering of data from national registries with high coverage rates, has in addition limited the amount of loss to follow-up and having a fixed end date rather than censoring on last medical visit has reduced potential bias, since medical contacts often are associated with the health status of the patient seeking care.

The risk classification of patients and presentation of data based on different subgroups of patients have also been important, since the underlying diagnosis for receiving GH treatment has a major impact on cancer risks. Moreover, the analysis by dose and duration categories has not been done in previous studies and is essential to investigate any associations between treatment exposure and cancer outcomes.

The major limitation of the study is the lack of an untreated control group. All interpretations of relative risks must always carefully consider the properties of the comparison group. The extremely high SMRs and SIRs of the patients with a previous cancer diagnosis were to be expected since the comparison group was the general population and it is difficult to assess to what degree, if any, GH treatment has contributed. Thus, the analyses with dose and duration as well as particular patterns of site-specific malignancies become essential.

Similarly, for the other risk groups of isolated growth failure, including GHD, ISS and SGA, or the other non-cancer diagnoses, unadjusted comparisons with a general population are less than ideal. These groups might as well have underlying factors associated with the outcome that could distort and confound the associations presented. However, such underlying factors could affect the risk in both ways, not only imply an inherent increased risk for the outcome but also a possible inherent decreased risk. An argument could perhaps be made that considering the known association with increased height<sup>295-297</sup> as well as increased IGF-I levels<sup>221,251,298,299</sup> and cancer risks, a short untreated group should *a priori* be expected to have a lower baseline cancer risk than the general population of taller stature. If this would be the case, then a similar risk as the background population after treatment would for these patient groups actually mean that their risk has increased from a hypothetically lower baseline risk.

Furthermore, in the low-risk group of isolated growth failure, severe diagnoses and previous cancer diagnoses have been filtered out but such diagnoses all remain in the comparison group of the general population, albeit diluted. Thus, when comparing a patient group where by definition no previous cancer may exist to a population where all childhood cancer still remains, even if rare and diluted as in the general population, those cases might still contribute to such a degree that the comparisons with a group in which such diagnoses have been filtered out becomes inaccurate.

There are also several other limitations to the study, such as the lack of GH treatment information beyond pediatric ages, lack of information on IGF-I levels, and still quite few individuals beyond the age of 35 years, restricting any conclusions of cancer risks in older ages. Studying rare events also creates issues with statistical power, as for study III, with more uncertainty in the results illustrated by wide confidence intervals. Lastly, including data from eight different countries creates a complexity to the aggregated dataset but, at the same time, also increases the external validity of the study and was an inevitable element of creating a large enough cohort to achieve sufficient statistical power for these analyses.

## 7 CONCLUSIONS AND FUTURE PERSPECTIVES

The main conclusions of each study are presented below:

### Study I

- Few overall metabolic differences were seen between short prepubertal children with stimulated GH peak levels of 7-14  $\mu\text{g/L}$  and healthy age- and sex-matched controls of normal height and weight.
- The subgroup of short prepubertal children with the lowest stimulated GH peak level ( $<10 \mu\text{g/L}$ ) differed from both the other short children and controls, with lower levels of fasting insulin and signs of increased insulin sensitivity.

### Study II

- A dose-dependent effect of GH treatment on several metabolic parameters was found in the short children from Study I and in particular for the high dose group (100  $\mu\text{g/kg/d}$ ) regarding fasting insulin levels and indices of insulin sensitivity. All groups showed decreased insulin sensitivity during the study but the low (11  $\mu\text{g/kg/d}$ ) and standard (33  $\mu\text{g/kg/d}$ ) dose groups remained within normal reference ranges for fasting insulin and HOMA-IR.
- The high dose group showed the largest height SDS increase and increased levels of IGF-I and also significantly raised fasting glucose levels and HbA1c over the two-year study period. Effects on fasting glucose and HbA1c were not seen in the low or standard dose group.

### Study III

- Childhood GH treatment due to GHD, SGA or ISS, was associated with higher adjusted hazard ratios for overall and severe cardiovascular events later in life. The elevated risks ranged from around half to double for the different treatment groups with the SGA group and females showing the highest increased risks.
- In relation to dose and duration of treatment, the highest relative risks were seen for those with the longest duration and the highest cumulative dose but no association was found between higher mean daily dose and increased cardiovascular risks. A significant trend was additionally found for treatment duration but not for cumulative or mean dose.

- The absolute risks of cardiovascular events were low in both patients and controls.

## **Study IV**

- Overall cancer mortality and incidence risks were highly related to the underlying diagnosis for GH treatment. For patients with a previous cancer diagnosis, markedly increased cancer mortality and incidence risks were found in GH-treated patients compared to the general population. For patients with GHD, ISS or SGA, no overall increased cancer mortality or incidence risks were seen. For other non-cancer diagnoses, such as Turner syndrome or multiple pituitary hormone deficiency, slightly increased overall cancer mortality and incidence risks compared with the general population were found.
- For the patients with no previous cancer diagnosis, a site-specific increased mortality risk of bone tumors was found in the GHD, ISS and SGA patients and a site-specific increased incidence risks for bone and bladder cancer in patients with other non-cancer diagnoses, although based on few cases.
- In GHD, ISS and SGA patients, a significant trend of higher cancer incidence was seen with longer follow-up since first treatment and duration of treatment but not with mean dose or regarding cancer mortality. In patients with an initial cancer diagnosis, higher mean daily dose was associated with increased cancer mortality but not with increased cancer incidence.
- For the site-specific analyses, longer follow-up was associated with a decreasing risk of CNS tumors in patients with an initial cancer diagnosis, and an increasing risk for Hodgkin's lymphoma in non-cancer patients.

## **Future perspectives and concluding remarks**

The results of the studies presented in this thesis provide some answers but also bring with them many new questions and several ideas of new projects within this field have emerged along the way, of which some will be presented below.

Regarding the metabolic characteristics in patients with different levels of activity in the GH/IGF-axis, an expansion of study I and II, also including patients with stimulated GH levels  $<7 \mu\text{g/L}$  would be of great interest. To characterize the metabolic profile over the whole spectrum of GH secretion would be valuable for our understanding of the metabolic properties of GH and GH treatment. Additionally, to further investigate the treatment effects, having a group of patients also randomized to no treatment, as a comparison group within

study II, would have been useful to better separate the physiological metabolic changes occurring in this age of transition into puberty from effects of various GH doses. With respect to the strong links with fasting insulin and insulin sensitivity found, analyzing other parameters, such as for example IGFBP1, known to be related to hepatic insulin sensitivity, could also have been valuable to improve our understanding of such a connection.

For study III and IV, the continuous surveillance and follow-up of these patient cohorts are required in order to have a more complete answer to the long-term safety of childhood GH treatment. Both cohorts are still quite young and what long-term risk that might occur in later adulthood and older ages are still uncertain. With older age, increasing number of cancer and cardiovascular events are expected, which would improve the statistical power and precision of future comparisons. Having information of more covariates of importance, such as IGF-I levels in the patient group, would also have been very valuable in order to investigate if cancer risks are related to IGF-I levels. Such information could also have important clinical implications in our dosing of GH to different patient groups.

For study IV, to further analyze cancer risks with more suitable comparison groups for the different patient groups would be of great value. Having a similar possibility to adjust for several possible confounders, as was done in study III, would improve the comparisons and would have created more informative results regarding the relative risks. To create a randomized trial with life-long follow-up would of course be ideal, but since this option is not available due to practical, ethical and economic reasons, our best alternative is well-designed observational studies.

As a concluding remark, the clear metabolic effects and the indications of possible future risks further emphasize the need of continuous follow-up of GH-treated patients so that our understanding and knowledge can continue to increase regarding the metabolic effects and long-term safety of childhood GH treatment.

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